DATE DUE

JAN 4 1973 MP			
GAYLORD			PRINTED IN U.S.A.

PLATE I

TYPES OF MICROÖRGANISMS PRODUCING DISEASES IN ANIMALS.

I.—Bacillus anthracis, sporulating; pure culture on agar, stained with fuchsin. The cause of anthrax in cattle and other domestic animals.

II.—Bacillus sarcophysematos bovis, sporulating; pure culture on glucose agar, stained with fuchsin. The cause of black-leg in cattle.

III.—Bacillus tetani, sporulating; pure culture in glucose bouillon, stained with fuchsin. The cause of lockjaw in the horse.

IV.—Bacillus mallei, pure culture on glycerin agar; stained with methylene blue. The cause of glanders in the horse.

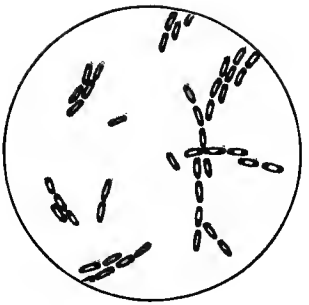
V.—Bacillus of tuberculosis from the gland of a hog. Stained with carbol-fuchsin.

VI.—Spirillum or vibrio of Metchnikoff, from a pure culture on agar, stained with fuchsin. The cause of vibrio cholera in the chicken.

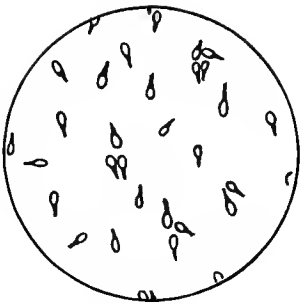
VII.—Two halteridia infecting the red blood corpuscles of a bird. A protozoan organism and the cause of avian malaria.

VIII.—A representative of the flagellate protozoan organism trypanosoma. The cause of surra, nagana, etc., in horses, cattle, and other domestic animals.

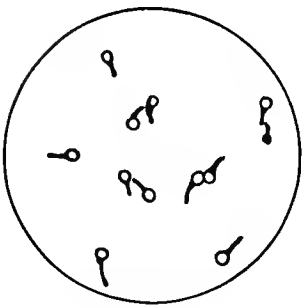
PLATE I



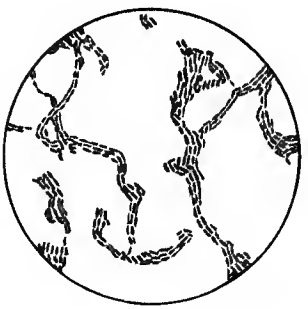
I.



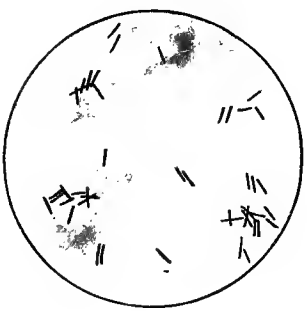
II.



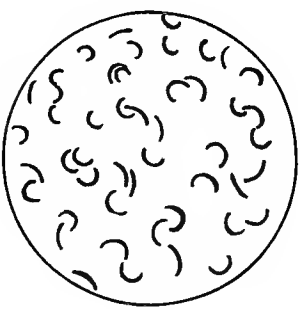
III.



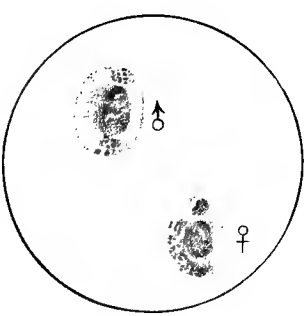
IV.



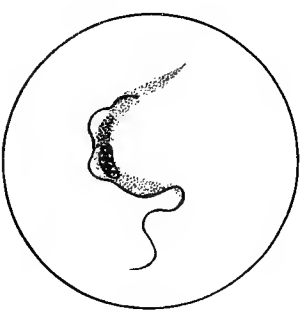
V.



VI.



VII.



VIII.

A TEXT-BOOK

ON

DISEASE-PRODUCING

MICROÖRGANISMS

ESPECIALLY INTENDED

FOR THE USE OF VETERINARY STUDENTS AND PRACTITIONERS

BY

MAXIMILIAN HERZOG, M.D.

PROFESSOR OF PATHOLOGY AND BACTERIOLOGY IN THE CHICAGO VETERINARY COLLEGE, PATHOLOGIST TO THE GERMAN AND THE ALEXIAN BROTHERS' HOSPITALS OF CHICAGO, LATE PATHOLOGIST IN THE BUREAU OF SCIENCE, MANILA, P. I., ETC.

WITH 214 ILLUSTRATIONS IN BLACK AND 14 COLORED PLATES

LEA & FEBIGER
PHILADELPHIA AND NEW YORK
1910

PREFACE

THERE does not exist, so far as the author knows, in the English language, any text-book on Pathogenic Microörganisms, written especially for the use of veterinary students and practitioners, nor are there many such books in any language. In fact, the only work of this kind with which the author has been familiar in the past is by Professor Th. Kitt, *Bakterienkunde und Pathologische Mikroscopie für Thierärzte und Studierende der Thier Medizin.* While lecturing and conducting the practical courses in pathology and bacteriology during the last few years in the Chicago Veterinary College, the author has been often approached by students with the request to furnish them with a manifold summary of the lectures and laboratory talks on the use of the different stains, culture methods, animal inoculations, etc. It was first during the course of 1909–10 that the author acceded to this demand and furnished to the students a mimeographed summary of the work in these departments. The result was satisfactory both to teacher and students, and the author, therefore, concluded to prepare a systematic text-book covering the needs of veterinary students and practitioners, with illustrations wherever desirable. He feels confident that such a publication will be of assistance both to the teachers of bacteriology in veterinary schools and to their students, and hopes that the book will find an interested circle of readers and consultants among veterinary practitioners. Many of these have been students when bacteriology was very insufficiently taught in most of the veterinary schools of our country, and now, when the importance of this subject has been so well recognized both in human and in veterinary medicine, they cannot help but feel the necessity of getting an elementary knowledge of the theory and practice of dealing with pathogenic microörganisms. In the consideration of the infectious diseases of animals most important in veterinary practice, the morbid anatomy and histopathology has also been fully covered in the following pages. It has been the constant endeavor of the author to be explicit and to introduce and develop the subject in such a manner that the book might be used for self-instruction by any reader who has already gained a moderate elementary knowledge of biology.

While the book is primarily intended for veterinary students and practitioners, it is hoped that it will also be of use to the medical student

who wishes to give attention to the comparative bacteriology of man and the domestic animals. It may also find a place in the curriculum of agricultural colleges where bacteriology is becoming more and more taken up, and where it is also especially studied with reference to veterinary science and to certain fermentative processes in the soil, and in milk and milk products, as butter, cheese, etc. Micro-organisms in relation to such processes have, therefore, been fully considered.

A somewhat novel feature for a book of this character has been introduced, namely, the addition after each subject or chapter of "Questions." A student may read a chapter once or even several times, and he may think that he has fully mastered and well remembers the subject. Yet when it comes to exercises in recitation in the class room or to a written examination he may fail. The author, therefore, believes that it will be of great advantage to the student to have after each chapter a number of questions covering the subject treated, and enabling him to put his knowledge to a test to find out whether or not he has mastered the task of committing to memory the main facts given. Such questions in a voluminous text-book or work of reference would, of course, be unnecessary, but they will serve a useful purpose in an elementary book for the beginner, whether he be student or practitioner.

The author has freely consulted the following works: Kolle and Wassermann, *Handbuch der Pathogenen Microörganismen;* Kitt, *Bakterienkunde und Pathologische Mikroscopie für Thierärzte;* Flügge, *Die Mikroörganismen;* Nocard and Leclainche, *Les Maladies Microbiennes des Animaux;* Moore, *The Pathology and Differential Diagnosis of Infectious Diseases of Animals;* Hutyra and Marek, *Specielle Pathologie und Therapie der Hausthiere;* Laveran, *Trypanosomes and Trypanosomiases;* Doflein, *Die Protozoen als Parasiten und Krankheitserreger;* Calkins, *Protozoölogy;* Lafar, *Technische Mykologie;* Kraus and Levaditi, *Handbuch der Technik und Methodik der Immunitätsforschung;* the publications of the Bureau of Animal Industry, United States Department of Agriculture, and those of the Bureau of Science, Manila, P. I.

M. H.

Chicago, 1910.

CONTENTS

PART I

THE THEORY AND PRACTICE OF GENERAL BACTERIOLOGY

CHAPTER XXXVIII

CHAPTER XXXIX

CHAPTER XL

CHAPTER XLI

PART III

MICROORGANISMS IN FOODS AND SOILS.

CHAPTER XLII

CHAPTER XLIII

CHAPTER XLIV

CHAPTER LII

CHAPTER LIII

CHAPTER LIV

CHAPTER LV

APPENDIX

PART I.

THE THEORY AND PRACTICE OF GENERAL BACTERIOLOGY.

CHAPTER I.

INTRODUCTORY HISTORICAL REVIEW.

THE sciences developed by mankind owe their early awakening and subsequent growth to two entirely different sets of motives. One of these is furnished by the necessities of life, in its everlasting struggle for existence; the other by that intense desire of the human race to unravel the mysteries of nature and solve the enigma of the origin of life.

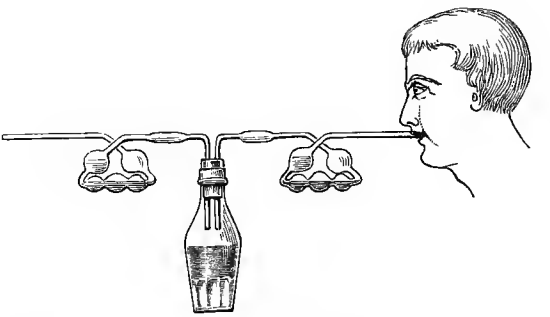
That side of modern medical science which deals with micro-organisms in relation to disease begins with attempts to recognize the true nature and cause of disease and with experiments to ascertain whether life can originate by *spontaneous generation*. The conception that many diseases are due to microörganisms originating in or invading the body, and multiplying therein, was first formulated long ago, to be forgotten, and to be taken up again with renewed vigor after the discovery that certain fermentative processes are due to these minute bodies. This discovery was itself stimulated by the long-continued experimental quarrel over the question whether or not spontaneous generation of life occurred in fermenting and putrefying organic materials.

That diseases of mankind might be due to forms of life so small as to be invisible was conceived as a purely hypothetical idea long before the compound microscope had been invented. Varo, in the first century before Christ, stated in writing that there might perhaps exist animals so small that they could not be seen, but that might enter the human body with the air through the mouth and nose and so produce disease. Nothing, of course, but a mere hypothesis in this direction could be formed before these microörganisms were seen.

The first combination of lenses was constructed by Hans and Zacharias Janssen, father and son, living in Holland in 1590. Their

instrument was evidently very poor and did not lead to any important discoveries. It was not until the seventeenth century that the microscope really gained importance, when Antony Van Leewenhoeck, the

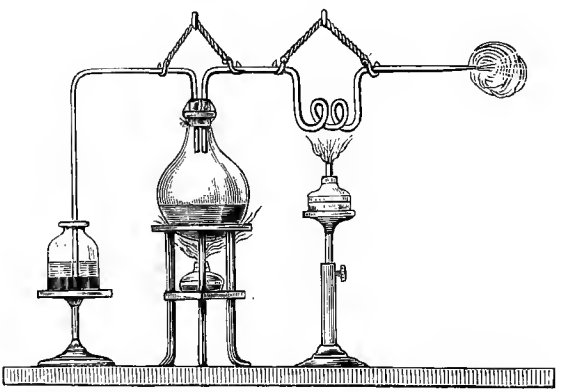
FIG. 1



Experiment of Schulze: Forcing air through sulphuric acid. (Lafar.)

true father of microscopy, succeeded in producing a fairly good instrument with magnification up to 150 diameters. He discovered the spermatozoa, and numerous small live organisms in saliva, stagnant water, fermenting and decomposing fluids, and organic materials.

FIG. 2



Experiment of Schwann: Heating air to make it sterile. (Lafar.)

After these small organisms had been studied for a number of years the theory was promulgated, particularly by Van Helmont and Needham, that these forms of life arose by spontaneous generation in such fluids as meat infusion, etc., even after they had been boiled. This view was contested by Spallanzani, who showed that when a meat infusion had been boiled three-quarters of an hour, and kept from

access by the air the development of microörganisms would not take place. It was then claimed by the adherents of the theory of spontaneous generation that the expulsion of the air by boiling and the arrangements which prevented it from reëntering also prevented spontaneous generation. Franz Schulze and Theodor Schwan then devised methods to permit air to enter after it had either passed through sulphuric acid or had been heated in a glass tube. Schwan also showed that when certain poisonous chemicals were added to the meat infusion, microörganisms were not developed. His experiments were the first to show the effects of what we now call *antiseptics*[1] upon microörganisms. Schroeder and Dusch allowed air to enter the vessels containing boiled organic substances through glass tubes which had been plugged with cotton. This method is now universally used in bacteriologic work to protect sterile culture media

FIG. 3

Pastsur bulb.

or pure cultures from contamination. The experiments of the last two investigators were, however, not all successful, and development sometimes occurred in their cotton-plugged glass vessels. The question of spontaneous generation was not definitely settled when Pasteur took it up before 1860. He showed, in the first place, that a short boiling of an infusion of organic material was not sufficient to kill all microörganisms, and that some could evidently withstand the temperature of boiling water for several hours. It was subsequently shown by the botanist F. Cohn and by Robert Koch that these resistant forms of microörganisms were the spores of bacteria.

[1] The term antiseptic in its strict sense means something which will prevent putrefaction or sepsis by inhibiting the growth of microörganisms, while the term germicide designates something that will kill germs or microbes. The word disinfectant is used synonymously with germicide. In practice most substances which act as antiseptics in a certain concentration will generally in a stronger concentration act as disinfectants or germicides. The term antiseptics, disinfectants, and germicides are used quite indiscriminately, and there is, indeed, between them no real generic difference but only one of degree.

Pasteur further constructed a peculiarly shaped glass receptacle, known now as the *Pasteur bulb*, which has a bent and curved neck through which air can freely circulate without, however, introducing microörganisms. In such bulbs meat infusions which had been boiled for several hours did not develop such growths. However, as soon as the neck was broken off, so that microörganisms could fall into the bulb, the fluid would decompose with the appearance of numerous microbes. The question of spontaneous generation was now settled, and it had been shown that microörganisms could not be so formed. In the meantime the cause of alcoholic fermentation of sugar-containing fluids had been discovered. Erxleben, as early as 1818, had made the statement that this fermentation was due to the multiplication and the metabolism of yeast cells. This, however, was not proved until about twenty years later by the extensive observations and experiments of Cagniard-Latour, Thedor Schwann and F. Kuetzing, who worked on the problem simultaneously. The vegetable nature of yeast cells had also been recognized, and the idea that diseases were due to vegetable and other microörganisms received a new stimulus. Athanasius Kirchner had already expressed this belief in 1659, and it was strongly upheld in the middle of the eighteenth century by Plenciz and Reimarus, the former believing that each infectious disease was due to a specific microörganism.

These theories had been long forgotten at the beginning of the nineteenth century; but after the *saccharomyces*, or *yeast cells*, had been recognized as the cause of fermentation, the microbic theory of disease was again revived, particularly by the celebrated German pathologist and anatomist Henle, who, in 1840, declared himself in favor of this theory. He was cautious enough, however, to state that it was not sufficient to find microörganisms in certain diseases, but that they must always be present in such cases, and further, that they must be shown actually capable of producing the disease. The succeeding years brought observations and discoveries which demonstrated the fact that bacteria are the cause of certain diseases. The anthrax bacillus was seen and later inoculated into animals by Polländer and Davine (1850–60). Rindfleisch, Recklinghausen, Waldeyer, and Klebs saw the pyogenic cocci in pyemia, puerperal sepsis, and wound infections. Robert Koch, in 1876, published his researches on the anthrax bacillus and two years later those on mouse septicemia. Bollinger, in 1878, recognized the significance of the ray fungus. About this time Robert Koch introduced the use of solid culture media for the purpose of isolating bacteria and obtaining them in pure cultures. In 1882 he published his researches on the tubercle bacillus. Kitasato later showed how to cultivate the anaërobic tetanus bacillus, and somewhat earlier the first disease-producing protozoa had been discovered. Griffith Evans, in 1880, saw in India, in the blood of horses, mules, and camels suffering from surra, a motile microörganism which he described as a spirillum. He

succeeded in producing the disease in healthy horses and dogs by inoculating the blood in which he had seen these organisms. Steele confirmed Evans' observations, and named this pathogenic organism *Spirocheta Evansii*. It is now known as *Trypanosoma Evansii*, and was the first pathogenic trypanosome to be discovered. In 1882 Laveran discovered the plasmodium malariæ, and early in the next decade Theobald Smith discovered the protozoön *piroplasma bigeminum* as the cause of Texas fever.

Since the fundamental work of Pasteur and Robert Koch, studies of pathogenic microörganisms in general and of pathogenic bacteria in particular have been placed on a firm basis, and have assumed the greatest importance in the theory and practice of human and veterinary medicine.

CHAPTER II.

ORGANISMS—SAPROPHYTES—PARASITES—GENERAL REMARKS ON DISEASE-PRODUCING MICROÖRGANISMS.

THERE are two classes of objects in nature, one alive and animated, the other one inanimate. To the latter belong rocks, minerals, chemicals, etc. If we examine an object belonging to this class, for instance a piece of iron, we find that any part is similar in structure to the whole block, and has all its properties. If, on the other hand, we examine a live object, in other words a living being, we soon notice that there are several, in fact many, parts unlike each other in properties, and, of course, individually unlike the whole, and that these different parts or portions perform different functions. Such different parts of a living being are called its *organs*, and the general scientific term for live objects is organisms. Modern studies have shown that all organisms are built up of small component parts called *cells*. Most organisms are composed of a multitude of cells, but there are many which consist of only a single cell. These are called *unicellular organisms*. Such very minute beings can be seen only with the aid of the microscope, hence they are known as *micro-organisms*, and, more popularly, often as *microbes*.

We divide organisms into *plants* and *animals*, and an enumeration of the differences between a higher plant, for instance a tree and a higher animal, and for instance a horse, is easy. However, when we come to the unicellular organisms of the lowest type it is sometimes difficult to decide whether we are dealing with a minute plant or a minute animal. There are, in fact, microörganisms which are classified as vegetables by some investigators and as animals by others.

Pathogenic Microörganisms.—The following pages will deal particularly with the microörganisms, both animal and vegetable, which may invade the body of man and domestic animals, and may there multiply, in this manner becoming the cause of the so-called infectious diseases. Such minute disease-producing organisms, or pathogenic microörganisms, belong to the various phyla, tribes, classes, orders, and families. The common feature which makes them interesting and important to the student and practitioner of human and veterinary medicine is the fact that they are the cause of much disease, suffering, loss, and death. It is well to point out in the beginning that while certain of these pathogenic microörganisms cause disease in man, and not in the lower animals, as, for instance, the microbes of typhoid fever, leprosy, syphilis, etc., and while others cause disease

in the lower animals only, as, for instance, the microörganisms of Texas fever of cattle, black-leg of cattle, diphtheria of calves, leg and lip diseases of sheep, etc., many other microörganisms cause identical or very similar diseases both in man and the lower animals. To these latter belong the pathogenic microörganisms which produce such common diseases as tuberculosis, actinomycosis, glanders, tetanus or lock-jaw, inflammations, suppurations, blood-poisoning, etc. Hence, studies concerned with disease-producing microörganisms in their relation to human and to veterinary medicine overlap, and the general underlying principles and the methods employed for the elucidation of the subject are identical. It is, of course, obvious that in a book primarily designed for veterinary students and practitioners, pathogenic microörganisms will be taken up especially with reference to diseases of the domestic animals. Reference to human diseases will only be made in a brief manner, in so far as is necessary to point out sufficiently certain common features and to emphasize the possibilities and the dangers of the transmission of diseases of domestic animals to human beings, and vice versa. This is a subject in which the veterinarian is interested both from a personal standpoint and on behalf of the community. In other words the modern scientific veterinarian must be a hygienist, not merely for the benefit of his patients, the domestic animals and their owners, but also for the benefit of mankind at large.

Non-pathogenic Microörganisms.—A limited number of microörganisms which are not disease-producers, and which, from a medical standpoint, are entirely harmless, will also be considered briefly. Such microörganisms are used in the laboratory training of the student to familiarize him with morphologic features and technical methods early in his studies, at a time when it would not be advisable to give into his unpractised hands dangerous, live, disease-producing microorganisms, and also because some harmless widespread bacteria are very similar to certain pathogenic bacteria. The student must learn to distinguish such harmless bacteria, as, for instance, the common hay bacillus or B. subtilis, from dangerous pathogenic bacteria like the anthrax bacillus, because they are very similar in their morphologic and cultural features.

Microörganisms Causing Fermentation.—Some microörganisms which are important in producing fermentative changes, both desirable and undesirable, in milk, cheese, and other organic materials, will also be briefly considered. These are also matters in which the veterinarian is likely to be consulted and in which he should be at least sufficiently well versed to form an intelligent valid opinion.

Microörganisms in Nature.—Microörganisms are, of course, not all disease producers; in fact, the great majority of them live in the outside world not merely a harmless but a very useful existence. Certain classes are the cause of necessary and useful fermentative processes; while others bring about the decomposition of dead organic

matter and split it up into simple chemical compounds, so that these can be utilized again in building up the higher plants, which in their turn are needed directly or indirectly to support the life of the higher animals. Without higher plants, herbivorous animals could not exist, and without the latter, the carnivora would perish. Certain microörganisms are, therefore, essential in maintaining the cycle of life on our planet. Microörganisms do not always live in the general outside world. They may be in or on other higher living beings, as parasites, and yet they may not do any harm to their host but may even benefit it and be a necessary element in its metabolism and existence. So it is incorrect to consider all microbes as enemies of mankind and domestic animals. It is true that some microorganisms are our greatest enemies, but many others are our greatest friends.

Saprophytes and Parasites.—Most microörganisms of both vegetable and animal types exist in the outside world. They derive their nutrition from dead organic material, which they split up in their metabolism, and they are known as saprophytes. The lowest microorganisms of a vegetable type, the saprophytic bacteria, exist almost everywhere on and near the surface of our earth. We find them in the air, in the water, in the soil, on the surface of the bodies of animals and plants, in decaying substances, etc.; they are, as it is also expressed, *ubiquitous.*

Organisms which live on or in a living host, and which utilize some of its material for their own nutrition, are known as parasites. Some microörganisms can live only as parasites, and can never thrive and multiply in the outside world, as, for instance, the microörganism causing tuberculosis, the tubercle bacillus, in its various forms. Such organisms are called *strict* or *obligate parasites.*

There are other microörganisms which can exist both as saprophytes and as parasites, such as those which cause anthrax in man and animals. This anthrax bacillus can exist and multiply on meadows, in manure, in the ground, and can also invade the blood of animals, where it greatly increases in numbers and causes the disease known as anthrax, or splenic fever. Microörganisms which can exist both as saprophytes and as parasites are called *facultative parasites* or *facultative saprophytes,* while those which can live only in the oustide world and never as parasites are called *strict* or *obligate saprophytes.*

When parasites live on the outside of their host, they are spoken of as *ectogenous parasites;* when they live inside the body, as *entogenous parasites.*

Commensales.—It must not be supposed that all parasites are harmful; many are perfectly harmless. These are called commensales.

The bacterium known as the colon bacillus is of this type and lives in the colon and other parts of the large intestines of man and

animals. It derives its nutrition from the contents of the large intestine, and is harmless except when it invades such organs as the gall-bladder, the urinary bladder, etc.

Symbiotes.—There are some parasitic microörganisms which are not only harmless but which are beneficial or even absolutely necessary. For instance, there are bacteria living on the roots of higher plants (clover) without which these plants could not obtain their nitrogen supply. Certain bacteria occur in the human and animal vagina which keep the secretion of the organ acid and tend to prevent disease-producing microörganisms from gaining entrance into the uterus. Such necessary or beneficial parasites are called symbiotes. The term *symbiosis* is, however, also used in bacteriology in a very different sense—namely, for two bactéria living together and producing by their united efforts a more virulent form of disease.

QUESTIONS.

1. What are the distinguishing characters between an animate and an inanimate object of nature?
2. What is an organ? What an organism?
3. What is a unicellular organism?
4. What is a microörganism or microbe?
5. What is a pathogenic microörganism?
6. What is an infectious disease?
7. Name some diseases which occur only in man and not in the domestic animals?
8. Name some diseases which occur in domestic animals and not in man.
9. Name some diseases common to man and to domestic animals.
10. Name a harmless non-pathogenic microörganism which looks very much like the anthrax bacillus.
11. What is the role of the microörganisms in general in nature? Why are they essential for the maintenance of life on our planet?
12. What is the meaning of the term saprophyte?
13. What is the meaning of the term parasite?
14. What does the term ubiquitous mean?
15. What is an obligate parasite? Name such a microörganism.
16. Is the anthrax bacillus an obligate parasite or not?
17. What is a facultative, what an obligate saprophyte?
18. What is an ectogenous parasite?
19. What is an entogenous parasite?
20. What is a commensale?
21. Name a bacillus which lives in the intestines of man and domestic animals as a commensale.
22. What are symbiotes?
23. What does the term symbiosis, when used in connection with pathogenic bacteria, generally signify?

CHAPTER III.

BACTERIA—GENERAL CONSIDERATIONS—MORPHOLOGY.

Definition.—Bacteria (singular bacterium) are very minute, unicellular, vegetable microörganisms, of round, cylindrical, or spiral shape, motile or immotile, which perform their nutritive function without the aid of chlorophyl, and which multiply very rapidly by binary division or fission.

Position among Organisms.—Bacteria have been placed as an intermediary phylum between animals and plants by some biologists and by others they have been classified as the lowest forms of animal life, because many are motile. If we consider their mode of nutrition without the aid of the chlorophyl of the higher plants and their whole metabolism we find that they are nearest related to the higher fungi. Their most logical classification is, therefore, among the plants. On account of their mode of multiplication by division they are also called *fission fungi*, or *schizomycetes*. Bacteria do not, like the higher plants, show any differentiation into root, stem, or leaves, but consist generally of a very simple single cell.

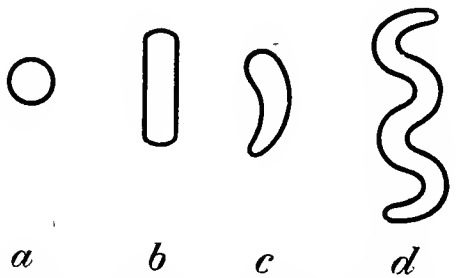
Types.—When bacteria were first studied by botanists and biologists in general (as, for instance, Naegeli and Zopf) it was believed that they were very variable in form, and that one and the same species might present itself alternately in ball-, rod-, or screw-form. The botanist F. Cohn was the first to claim that this was an erroneous impression, and that bacteria, like higher plants, were constant in shape. This was proved beyond doubt by Robert Koch and his followers. For the purpose of obtaining so-called pure cultures of a single species of bacteria, Koch for the first time devised and used solid culture media, which has enabled us to show beyond doubt that bacteria are constant in form, and that each type only reproduces itself. Three main types of bacteria are distinguished:

1. The coccus (plural cocci).
2. The bacillus (plural bacilli).
3. The spirillum (plural spirilla).

The *coccus* is a ball or spherical-shaped bacterium. The *bacillus* is a cylindrical rod-shaped bacterium very much like a short, round lead pencil. The *spirillum* is spiral or corkscrew-like in shape. It may consist of a portion of a screw winding, or it may show several twists, giving it the appearance of a complete corkscrew. In the former case we speak of a *vibrio*, while in the latter we designate the complete spiral as a *spirillum* (plural *spirilla*), or, better still, as a *spirochete* (plural *spirochetæ*).

These various types in propagation or multiplication always reproduce their own type, but it must be stated that under varying conditions bacteria sometimes vary from their most typical shape. For instance, the cause of pneumonia in man and animals is a double

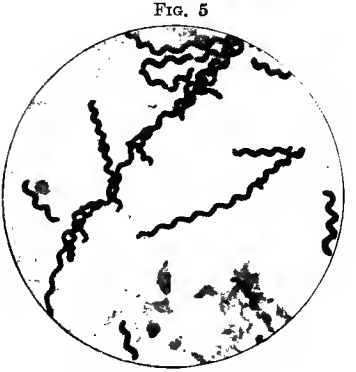
FIG. 4



Types of bacteria (schematic): *a*, coccus; *b*, bacillus; *c*, vibrio; *d*, spirillum.

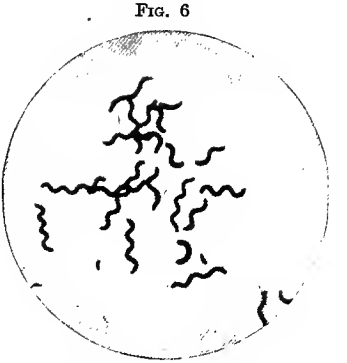
coccus, a so-called *diplococcus*, which in artificial cultures sometimes becomes elongated, so that it is lancet-shaped instead of ball-shaped. Other cocci may, under certain conditions, become flattened, so that they look like a half-moon or somewhat like a crescent. Sometimes bacilli in multiplying may become very short, so that they look on

FIG. 5



Very large spirilla. (Park.)

FIG. 6

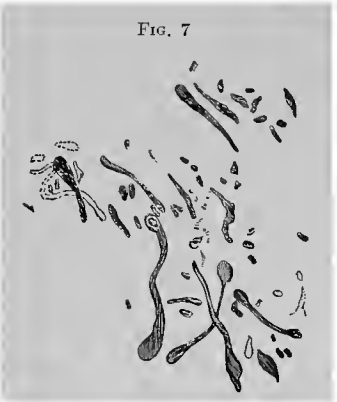


Medium-sized spirilla.

superficial examination like cocci. The three kinds named, however, never change their shape permanently or so completely that they really assume another type.

Deviation (Pleomorphous Bacteria).—Some bacteria, however, may show, under certain conditions, not always well understood, certain deviations from the common normal or average type. They may, for

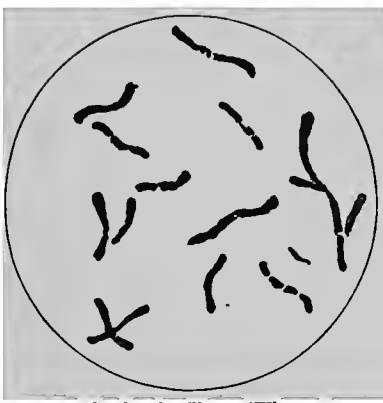
Fig. 7



Involution forms from bacilli. (From Flügge.)

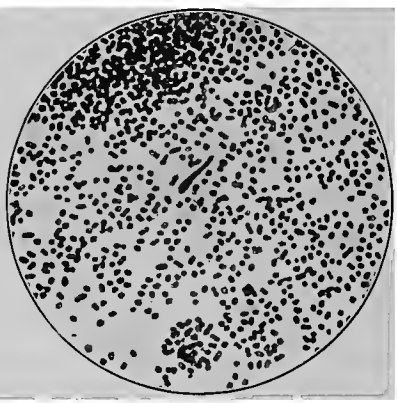
instance, form true branches. The tubercle bacillus, the glanders bacillus, and the diphtheria bacillus belong to that type of bacteria which form occasionally true branches commonly found among higher microörganisms of a vegetable type, namely, the moulds. Bacteria which in this respect deviate from the normal or average type are called pleomorphous bacteria. Other bacteria, as, for instance, the Bacillus proteus vulgaris, may form *pseudobranches*, that is, false branches due merely to an arrangement which resembles the preceding type. Such pseudo-

Fig. 8



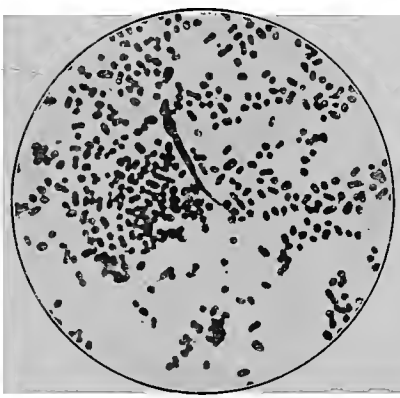
Glanders bacillus. (Wherry.)

Fig. 9



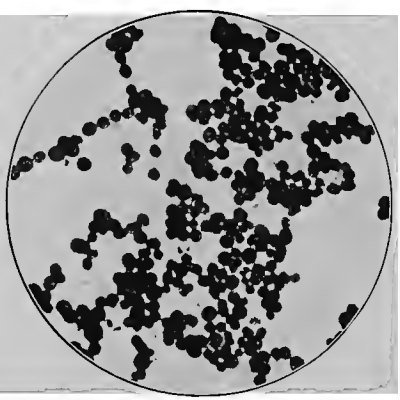
Bacillus of bubonic plague. (Herzog.)

Fig. 10



Bacillus of bubonic plague. (Herzog.)

Fig. 11



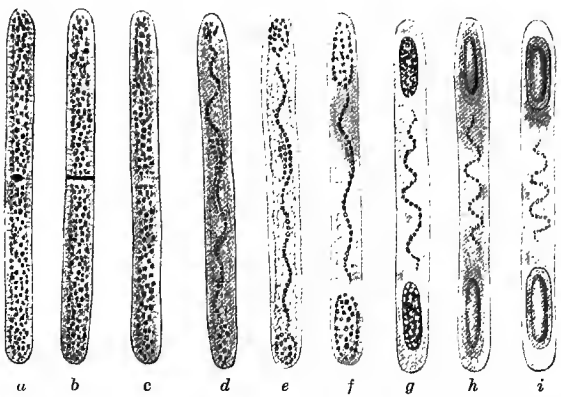
Bacillus of bubonic plague. (Herzog.)

Figs. 8 to 11 illustrate various types of involution forms.

branches are formed in a chain of bacilli when one near the centre multiplies, and in doing so pushes the new bacillus formed out of the line, so that it projects to either side, but still retains its connection with the bacillus which produced it.

Involution Forms.—Bacteria often show very abnormal forms under unfavorable conditions of growth, but these must simply be looked upon as degenerates, or cripples. They are known as involution forms. For example, the coccus of pneumonia on artificial culture media shows long, irregular, bacilli-like forms; the anthrax bacillus, which is a stiff, straight, cylindrical rod, becomes curved; the plague bacillus in old cultures forms spermatozoa-like bodies, and the same bacillus on an artificial culture medium (agar), containing 3 to 4 per cent. of common salt, forms large, round, yeast, cell-like balls. In very old cultures bacteria undergo splitting up or frag-

FIG. 12



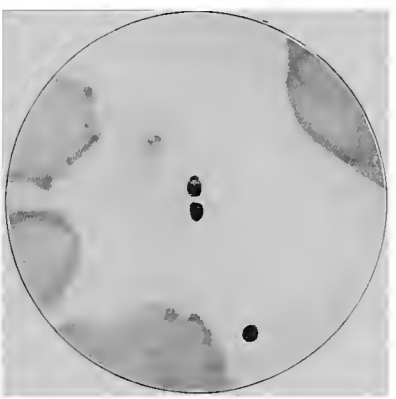
Bacillus Bütschli: *a* to c, incomplete division of the cell; *d* to *f*, gradual collection of chromatin granules at ends of cells; *g* to *i*, formation of end spores from these chromatin end-masses. (After Schaudinn.)

mentation, and may finally break up into irregular granules. However, as soon as such involution forms are placed in fresh, good culture media, under favorable conditions, they assume again their normal shape, with all of their normal average properties. It is only when unfavorable conditions are present, such as exhaustion of the soils, accumulation of metabolic products, etc., that the foregoing changes take place, otherwise bacteria always reproduce their own type and are not polymorphous. Involution forms are, as a rule, live bacteria, and if they are pathogenic they can produce their specific disease. If inoculated into a fresh culture soil they will reproduce the normal type of the species.

Bacteria can be killed with chloroform or formalin vapors in such a manner that they retain their shape perfectly. This should be done when cultures of dangerous bacteria are placed in the hands of beginners in the laboratory study of pathogenic microörganisms.

Cell Structure.—The cell which forms the body of a bacterium does not show any well-marked differentiation into a protoplasmic body and a nucleus. Most of the substance of the bacterium, however, takes the so-called nuclear stains, particularly the basic anilin stains, and extensive studies have shown that most of the body consists of diffusely distributed nuclear substance called *chromatin*. The latter is not contained in a well-defined nuclear membrane, as in cells of higher plants and animals, but is intimately mixed with a scanty amount of protoplasm called the *entoplasm*, around which there is often a very small rim of *ectoplasm*. In certain very young bacteria, or in bacteria which are at rest and not dividing a small nucleus-like body, can sometimes be demonstrated, but in all actively dividing bacteria the chromatin fills the interior of the cell and is present to such an extent that it almost completely hides the scanty amount of entoplasm.

FIG. 13



Postmortem smear from the heart blood in a case of bubonic plague, showing one plague bacillus in the centre of the field, with polar bodies stained very deeply. × 2000. (Author's preparation.)

Bacteria often contain distinct granules which in the unstained condition are highly refractive, and when stained take the dye in a very intense manner. These granules are known as the *metachromatic bodies* or the *polar bodies*, since they are found at one or both ends of a bacillus. They are also called the *Babes-Ernst granules*, after the two investigators who first described them minutely. These polar bodies must not be confounded with the *sporogenous granules* (see below).

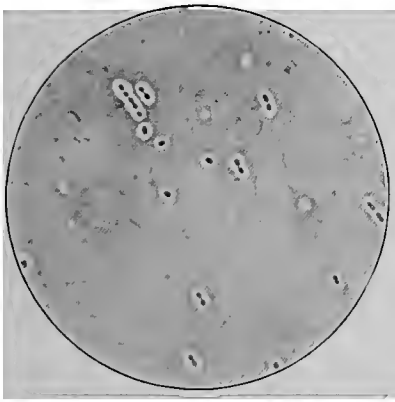
The ectoplasm of the bacteria does not usually stain by the ordinary methods used. While generally scanty, the ectoplasm may be more powerful, particularly in bacteria with many *flagella* (see below). It is believed, and perhaps fairly well demonstrated, that bacteria generally possess a membrane between the ectoplasm and the endoplasm, and, as a rule, some, under definite conditions, possess outside of the ectoplasm a smaller or larger gelatinous capsule surrounding the bacteria. The jelly-like masses forming these capsules may become confluent, and so form one *gelatinous matrix* in which the bacteria are embedded like cells of higher animals in an intercellular substance. Such formations are known as *zoöglœa* or *zoöglœal masses*.

Flagella.—If we study various bacteria in the live state in a drop of water or other suitable fluid we will notice some that possess the

power of locomotion. These *motile bacteria,* when observed in fluid, shoot and dart about like a school of minnows in clear water. Bacteria which are truly motile possess organs of locomotion in the shape of exceedingly fine slender threads or filaments called flagella (singular flagellum). These filaments are found at one or both ends, or all around the body. Generally, only bacilli and spirilla possess flagella, but a very few cocci also have them. We classify flagellate bacteria as follows:

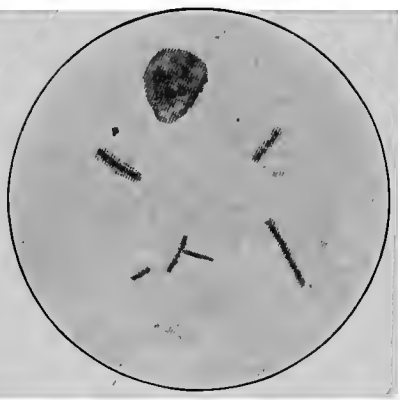
Monotricha—one flagellum at one end.
Amphitricha—one flagellum at each end.
Lophotricha—several flagella at one end.
Peritricha—flagella all around the bacterium.
Atricha—no flagella.

FIG. 14



Fraenkel's pneumococcus, pure culture in litmus milk, showing capsule. × 1000. (Author's preparation.)

FIG. 15



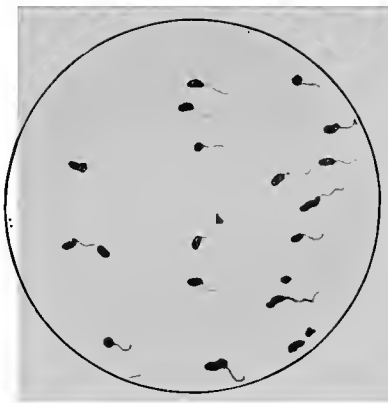
Anthrax bacillus in the blood of an infected cow, showing capsule. × 1000. (From a preparation of Dr. L. E. Day.)

Characteristics.—Flagella are, as a rule, exceedingly *slender filaments,* several times longer than the bacterium itself. They are wavy in outline and terminate in a blunt or slightly club-shaped extremity. They cannot be stained by the ordinary bacterial staining methods, but require a special complicated technic. They break off easily from the bacterium, but as far as known they are easily regenerated. They arise out of the ectoplasm, but are probably also connected with the entoplasm.

Brownian Movement.—Even those bacteria which have no flagella, and which in consequence have no true locomotion when examined in a drop of water, present a peculiar oscillating, trembling motion, but this movement is not confined to living bacteria. Dead bacteria or very minute particles of solid matter, when suspended in fluid, show a similar motion, which is called the Brownian movement, because it was first described by the botanist Brown. It is now

known that this motion of very small solid particles in fluid is due to the fact that the adjacent particles of gaseous or liquid matter surrounding them are in constant violent motion, and these lively

Fig. 16



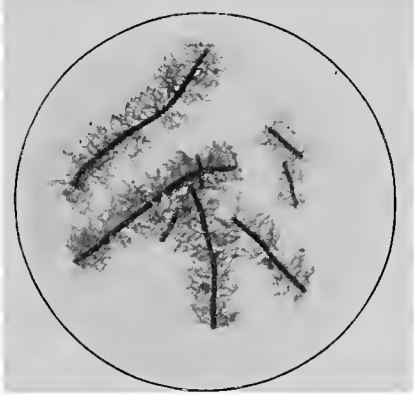
Spirillum of Asiatic cholera, showing single flagellum. (Kolle and Zetnow.)

Fig. 17

Spirillum volutans, showing flagella at either end of the bacterium.

molecules bumping constantly against the small, not truly motile bacteria keep them in a continued state of trembling agitation. It was formerly believed that the Brownian movement depended upon surface tension, but this is not the case.

Fig. 18



Bacillus proteus vulgaris, showing numerous flagella around the entire body of the bacterium.

Multiplication or Propagation.—Under suitable conditions bacteria take up nutritive material and multiply very quickly. This process is so rapid that a single bacterium, if circumstances are favorable, may in twenty-four hours have increased to many millions. It has been ascertained that several of the disease-producing bacteria under the most favorable conditions divide once in less than thirty minutes.

Binary Division or Fission.—It is characteristic of bacteria that they always multiply by dividing in the middle and the elongated forms—namely, bacilli and spirilla always divide at right angles to their long axis. This mode of multiplication is called binary division, or fission, and often causes bacteria to group themselves in a very characteristic manner,

which has given rise to a subdivision of the cocci into several groups.

Subdivision of Cocci.—Cocci, after the first division, may adhere to each other, but after the second division usually separate, generally forming in groups of two, called *diplococci.* They may, however, adhere together until after the second division, in which case they form groups of four, called *tetrads* (tetra—Greek word for four). Again they may divide in all three directions of space and adhere together, then we obtain square packages of cocci called *sarcina.* Yet, again, division may be in one direction only, when there are formed regular rows or chains or cocci, called *streptococci* (chain-cocci). Finally, the division of the cocci may go on in an irregular manner in all three dimensions of space, resulting in the formation of irregular clusters, resembling bunches of grapes, which are called *staphylococci.*

FIG. 19

Varieties of spherical forms: *a*, tendency to lancet-shape; *b*, tendency to coffee-bean shape; *c*, in packets; *d*, in tetrads; *e*, in chains; *f*, in irregular masses. × 1000. (After Flügge.)

Classification of Cocci.—The single coccus, or micrococcus.

The diplococcus, or group of two cocci.

The tetrad, or group of four cocci.

The sarcina, or cuboidal (dice-like) group of eight or sixteen or more cocci.

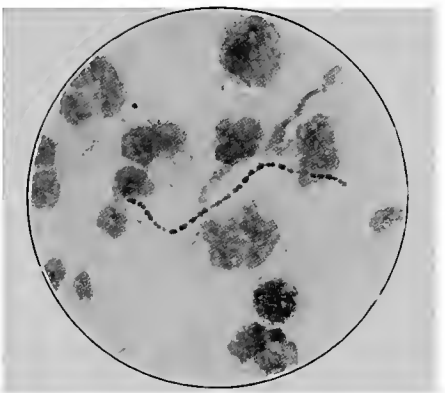
The staphylococci, or several cocci irregularly arranged like a bunch of grapes.

The streptococci, or a group of several cocci arranged like a row of beads (chain cocci).

Shape and Arrangement of Bacilli.—Bacilli shows varying features as to details of shape. Some are quite slender, others plump, thick, and short. Some of them, like the anthrax bacillus, are cylindrical, straight, and stiff, with square ends, while others show rounded ends, like the typhoid, colon, and hog-cholera bacilli. There are bacilli which are pointed at one end and club-shaped at the other, or club-shaped at both ends, like the diphtheria and glanders bacilli. Again, others, instead of being straight, are often slightly but distinctly curved like the tubercle bacillus. Certain bacilli, as the anthrax,

typhoid, colon, and hog cholera, have a tendency to form long chains; others, like the tetanus, group themselves in short chains of two or

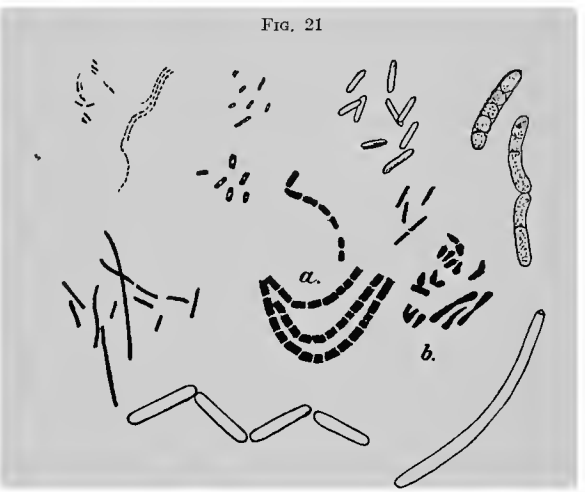
FIG. 20



Streptococcus in pus, showing how the chain is formed by groups of two (diplococci). The cells seen in the field are polynuclear leukocytes, with the exception of one cell, which is a mononuclear leukocyte. × 1000. (Author's preparation.)

three. Still other bacilli form long chains in which the lines of division between the individual bacilli cannot be seen, so that we see *pseudofilaments*. Certain bacilli rarely if ever form chains, but

FIG. 21



Various forms of bacilli: *a*, bacilli with sides parallel to their long axis and with ends perpendicular; *b*, bacilli with sides swollen or narrowed, causing irregular forms. × 1000. (After Flügge.)

generally fall apart after division and form parallel groups. When bacilli are studied in stained preparations some appear very uni-

formly colored, others stain in such a manner that dyed portions alternate with undyed sections of the entoplasm. Some bacilli take the stains easy, others with difficulty.

Size of Bacteria.—Microscopic Measure.—Bacteria are, as a rule, exceedingly small in size, their longest diameter being only a fraction of the diameter of a mammalian red blood corpuscle, and they can be studied individually only by the aid of good compound microscopes.

The size of bacteria is expressed by a microscopic measure based upon the metric system:

1 meter (about forty inches) = 100 centimeters = 1000 millimeters.

1 millimeter = 1000 micromillimeters.

1 micromillimeter or micron = about $\frac{1}{25000}$ inch.

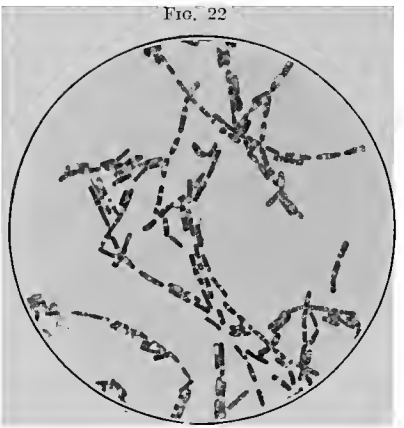
The term micromillimeter is indicated by the Greek letter μ or abbreviated micron (plural micra).

Sporulation or Spore Formation.—Under certain conditions, particularly when the soil in which bacteria have grown abundantly becomes exhausted, and when the metabolic products have accumulated, spore formation occurs. The spore of a bacterium may be likened in a certain sense to the seed of a higher plant. Only a limited number of the disease-producing bacteria form spores, and these are nearly all bacilli, very rarely cocci and spirilla. Spore formation is sometimes dependent upon very definite conditions, for instance, the anthrax bacillus requires the presence of free oxygen. Because these spores are formed in the interior of the bacterium they are known as *endospores*. Bacteria do *not multiply* by sporulation, since there is only one spore formed. However, this is not an absolute rule; exceptionally two spores have been found in one bacillus, but this occurrence is so very rare that it may be neglected entirely for any practical consideration. Spore formation is very important in the life history of bacteria, because the spores are very resistant to external inimical influences, and can stand antiseptics and heat very much better than the full-grown or *vegetative form* of the bacterium. In fact, some spores, as, for instance, those of the bacilli of tetanus, malignant edema, black-leg, and some soil bacteria, represent the most resistant organisms known. In order to kill tetanus spores with certainty they must be exposed for over an hour to the temperature of boiling water or steam at 100° C. This great resistance enables spores to survive where the adult vegetative form of the bacterium would perish. On account of their great resistance, spores in German are known as *Dauerformen*, which means *durable forms* of the bacterium. The great resistance of spores is largely due to the fact that they possess a very firm, tough, protecting membrane. In shape they are either round or oval, and are situated at either the centre or at or near one of the ends of the bacterium. Spores situated at one end of the bacterium, like that of the tetanus bacillus, give to the small rod the appearance of a drumstick. Spores situated in the centre, and making this part bulge out,

give the bacillus a somewhat barrel-shaped appearance. Such a bacillus is known as a *clostridium*.

Sporogenous Granules.—When spore formation is about to occur in a bacterium there appears in its interior, first, a dust-like transformation of the protoplasm, which gives it a powdered appearance; next appears one or more highly refractive bodies, the so-called sporogenous granules. These become confluent, and from them the spore is formed as a highly refractive body, composed of condensed protoplasm, and surrounded by a very firm, tenacious, tough membrane. The spore may escape from the bacillus at one end or it may rupture the bacterium in the equatorial plane. When a spore is placed under favorable conditions it takes up food material, its capsule ruptures,

FIG. 22



Bacillus subtilis sporulating. The unstained spaces in the centre of the rod are spores. (Author's preparation.)

and from its protoplasm is formed the ordinary, typical, vegetative variety of the bacterium from which the spore was originally formed. This process of the formation of the adult vegetative form of the bacterium from its spore is called the *germination of the spore*.

Arthrospores.—Cocci sometimes appear to change their whole body into a spore. These supposed spores were called arthrospores. Our knowledge of this type is limited and requires further study, but it is thought that the so-called arthrospores are merely involution forms. Of the disease-producing bacteria we may name as examples of spore-formers the bacilli of anthrax, tetanus, malignant edema, emphysematous anthrax, or black-leg; of harmless saprophytes the Bacillus subtilis and the Bacillus megatherium. Spores cannot be stained by the ordinary methods used to dye the vegetative forms.

QUESTIONS.

1. What unicellular vegetable microörganisms are classified as bacteria?
2. Why are bacteria best classified as plants?
3. Why are they called fission fungi, or schizomycetes?
4. Do bacteria in the course of their multiplication change their shape?
5. What are the three main morphologic types of bacteria? Name and describe them.
6. What method has enabled us to establish the constancy of the morphologic features of bacteria? Who devised this method?
7. What is the difference between a vibrio and a spirochete?
8. Do bacteria in their multiplication under variable conditions vary at all, and if so under what conditions?
9. What is meant by pleomorphous bacteria? Describe their characteristics and name some.
10. What is meant by a pseudobranching of multiplying bacteria? Name an example.
11. What are involution forms of bacteria? Under what conditions are they formed? Give some examples.
12. Are all involution forms dead organisms? What becomes of the involution forms when they are inoculated into a fresh culture soil?
13. How are bacteria killed so that they retain their normal morphologic features without degenerating into involution forms?
14. Describe the finer structure of the bacterial cell.
15. How is the chromatic substance of the bacterium arranged?
16. What is the entoplasm, the ectoplasm, the membrane of a bacterium?
17. What is meant by the metachromatic granules of a bacterium? By what other names are they also designated?
18. What is meant by the gelatinous capsule of a bacterium? What by a zoögloa or a zoögloal mass?
19. What is the difference between a motile and a non-motile bacterium? Have the former any organs of locomotion? How called? Describe in detail these organs of locomotion.
20. How are bacteria classified according to the number and arrangement of their flagella.
21. What types of bacteria generally possess flagella?
22. What is meant by the Brownian movement of a bacterium? What is it due to?
23. Name and describe the various types of cocci which result from the various modes of fission which occur in these ball-shaped bacteria.
24. Name and describe various morphologic types of bacilli.
25. What is a streptobacillus?
26. What are pseudofilaments?
27. What measure is employed to express the size of bacteria? What is the meaning of the Greek letter μ in bacteriologic nomenclature? What is the meaning of the terms micron and micra?
28. What is a bacterial spore? Describe its appearance.
29. Why is a bacterial spore called entogenous?
30. Does spore formation occur in all bacteria and under all conditions? If not, in what kind of bacteria does it occur, and when?
31. Name some disease-producing, spore-forming bacteria.
32. How many spores does a bacterium form?
33. What occurs in a bacterium during spore formation? Describe the phenomena in detail. Give location of spores in bacilli.
34. What is a clostridium?
35. Why is a spore called a "Dauerform" (durable form of the bacterium)?
36. What is meant by the vegetative form of a bacterium?
37. How and when do spores change into the vegetative forms of their respective species.
38. What is meant by sporulation? What by germination?

CHAPTER IV.

BIOLOGY OF BACTERIA.

In the previous chapter the morphology of bacteria has been considered. It is, however, impossible strictly to separate the morphology from the biology of these organisms. Involution forms and spores are certainly morphologic features of bacteria, yet they cannot be referred to without going to some extent into the biology of the fission fungi. The present chapter will be more particularly devoted to some important features in the life history of bacteria, as they are dependent upon varying conditions of their existence with reference to environments, nutrition, metabolism, etc. It has previously been stated that bacteria are as a class ubiquitous, that is, they are found everywhere on or near the surface of our globe, in soil, water, air, or the external surface of the bodies of plants and animals, and in the intestinal tract of the latter. They are not found in the blood or interior of tissues of healthy animals, nor to any extent in the highest altitudes and latitudes.

Temperature Limits.—A most remarkable feature of bacterial life in general is that they as a class can exist and multiply under a wider range of temperature than any other class of organisms. There are bacteria that multiply in sea water at 0° C. (32° F.) and others that multiply in springs at 75° C. (167° F.). Many individual species exist and multiply under a wide range of temperature, but some, particularly the pathogenic strict parasites of warm-blooded animals, are quite limited in the latitude of temperature under which they can exist and grow. Bacteria, as a rule, flourish and multiply most rapidly at a definite temperature called their *optimum temperature*. Beyond certain limits above and below the optimum temperature they will not grow at all; these limits are called the *maximum* and *minimum temperature* of their growth. The bacillus of mammalian tuberculosis has its optimum temperature at 37° to 38° C., that of avian tuberculosis at 38° to 43° C.; the former its minimum temperature at 29° C., the latter at 35° C.; while their maximum temperatures are 41° C. and 46° C., respectively. These are examples of strictly parasitic bacilli which have a very narrow range of temperature at which they can grow and multiply. This is also true of the strict saprophytes. On the other hand, facultative parasites, which occur also as saprophytes, often have the wide range of 25° C. from 15° to 40° C., and more.

Thermophile Microörganisms.—Bacteria which multiply best at very high temperatures (75° C.) are called *thermophile*, which, literally

translated, means heat-loving. They probably first appeared on our planet at a time when its surface was considerably warmer than it is now. They rarely multiply at temperatures below 40° to 50° C., and are found in abundance only in the tropics, in hot springs, and in soil which has been exposed to the direct rays of the sun for some time. They are quite common in the intestinal contents of animals.

Thermophile bacteria probably multiply considerably in spontaneously fermenting manure, and assist in bringing about by their metabolism the marked elevation of temperature. Some thermophile bacteria, in the absence of free oxygen, may be able to multiply at temperatures as low as 34° C. Such bacteria may possibly multiply in the intestines of man and domestic animals.

Thermotolerant Microörganisms.—There are some bacteria which multiply at quite high temperatures, but which have their optimum at 35° to 37° C. These are called *thermotolerant* (heat-tolerating). Bacteria in their vegetative form, with the exception of the thermophile and thermotolerant, are, as a rule, not very resistant to heat. They are generally killed at 55° to 60° C. if exposed in the moist state to this temperature for about ten minutes. The exact temperature and time has to be ascertained experimentally for each species, and again for the spores of such species as sporulate. This temperature when applied for a few minutes (generally five or ten) is called the *thermal death point* of the bacterium or of its spore. While heat easily damages the vegetative form of the bacteria, cold, as a rule, has very little effect upon bacteria and their spores. They may be frozen at a very low temperature without any effect, and will be found alive after thawing. However, repeated freezing and thawing in rapid succession kills some pathogenic bacteria.

Nutrition of Bacteria.—All bacteria depend for their existence and multiplication upon certain food materials and moisture. Many of them may be dried out completely without being killed, but they cannot multiply under such conditions, and only do so after they have again had access to moisture. Other bacteria when dried die very soon, as, for instance, the glanders and the plague bacilli. Spores of certain pathogenic bacteria, like those of anthrax, tetanus, blackleg, etc., can exist for years in a dried condition, and when conditions become favorable, germinate and display all of their general typical and special pathogenic properties. Some bacteria can exist for a long time in their vegetative form in a desiccated state, as, for instance, the tubercle bacillus. Desiccated bacteria and their spores must be considered as being in a condition where there is no metabolism and where life is latent, somewhat like life in higher plants in winter or in hibernating animals.

When bacteria grow in artificial cultures they exhaust the soil within a certain time, which, together with the accumulation of their metabolic products, produce conditions destructive to most of them. They die sooner if they are raised in the incubator than if raised at

room temperature. Bacteria in cultures may be kept alive much longer if they are tightly sealed up and placed in the refrigerator after the growth has been well developed. This not only keeps them alive much longer, but prevents, to a large extent, the appearance of involution forms.

Elements Necessary for Growth.—Bacteria need for their nutrition, growth, and multiplication substances containing the elements carbon, nitrogen, hydrogen, oxygen, sulphur, and phosphorus, and some salts. They can generally best derive their nutrition from albumins and their derivatives, like peptones and gelatins, but even pathogenic bacteria may be grown in albumin-free culture soils composed of very simple compounds. On the other hand, certain strictly parasitic pathogenic bacteria do not thrive well on artificial culture media, particularly during the first generations. The bovine tubercle bacillus is one of this type. Other bacteria have never been successfully cultivated, as, for instance, the leprosy bacillus or the acid-fast bacillus, which is found in such enormous numbers in Johne's disease of cattle. Other pathogenic bacteria grow only after the addition of unaltered hemogoblin to the culture soil, for example, the influenza bacillus. Still others require the addition of natural serous exudates, such as pleuritic or ascitic fluid. Too great a concentration of the culture soil with a high percentage of solids results in a poor growth or prevents it entirely.

Reaction of the Medium.—All bacteria are rather particular about the reaction of the medium in which they grow. Some favor a neutral, others a slightly alkaline, still others a slightly acid medium. Care must be taken in preparing the medium, as bacteria generally will not thrive if it is more than slightly acid or alkaline, and if there is any marked degree of either the bacteria will die, especially in the presence of mineral acids.

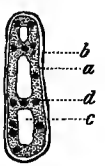
Plasmolysis and Plasmoptysis.—Many bacteria cannot stand the sudden transfer from one fluid to another, differing materially in concentration. Such sudden changes of osmotic pressure may cause a shrinking of the contents of the bacterial cell away from the membrane with a loss of fluid to the outside liquid; this is called plasmolysis. Or some of the cell contents may become expelled through a rupture in the membrane; this is known as plasmoptysis. These damaged bacteria are, however, not necessarily dead, and they may return to the normal if placed again under favorable conditions.

Aërobic and Anaërobic Bacteria.—Certain bacteria like the higher plants and animals can only exist and grow in the presence of free oxygen. Such bacteria are called *obligate* or *strict aërobes*. To this type belong many saprophytes, and among them particularly the color or pigment-forming so-called *chromogenic bacteria*, also some microörganisms which form poisonous decomposition products in milk. Among the pathogenic bacteria, the plague bacillus, the influenza bacillus, the pneumococcus, etc., belong to this group.

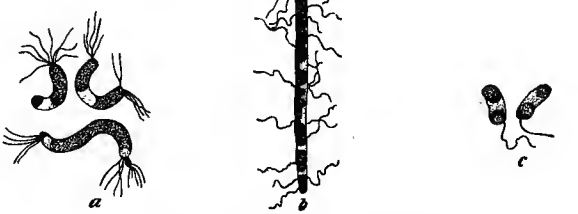
The opposite type of bacteria is represented by the *obligate* or *strict anaërobes*. These cannot exist and multiply in the presence of free oxygen, hence when raised in artificial cultures the air must be excluded or at least its oxygen. The pure nitrogen present does not, as a rule, interfere with the growth of the bacteria. Among the pathogenic bacteria the most important obligate or strict anaërobics are the bacilli of tetanus, black-leg, malignant edema, and the Bacillus necrophorus. Although strictly anaërobic bacteria die sooner or later in the presence of free oxygen, their spores may be exposed a long time before being killed. Strict anaërobes may, however, exist, grow, and multiply in the presence of free oxygen when they are intimately associated with aërobic bacteria, which in their growth use up and remove the oxygen present.

Facultative aërobes or *anaërobes* are those bacteria which can thrive in the presence or absence of free oxygen. To these belong many saprophytes and most pathogenic bacteria, such as the organisms of anthrax, of typhoid, of cholera, the pus-producing cocci, etc.

FIG. 23



Structure of bacterial cell: *a*, endoplasm; *b*, ectoplasm, or cell membrane; *c*, central, less intensely staining parts; *d*, chromatin granules. (After Bütschli.)



Plasmolysis: *a*, spirillum undula; *b*, bacillus solmsii; *c*, vibrio cholerae. (After A. Fischer.)

Metabolic Products.—Bacteria in their growth and metabolism excrete certain gaseous and soluble waste products: among them are carbon dioxide (CO_2), volatile compounds of nitrogen, such as NH_3, also free nitrogen, hydrogen sulphide (H_2S), etc. The latter is formed in all putrefactive processes depending upon bacteria in the absence of oxygen. Some bacteria in the presence of peptone form indol, for instance the colon bacillus, and this product may be used in differentiating nearly related bacteria from each other (the typhoid from the colon bacillus). Indol may be formed in consequence of putrefactive processes or by chemical decompositions of a different type. Other chemical reactions brought about by pathogenic bacteria are the reduction of reducible substances with oxygen absorption on the part of the bacteria, reduction of nitrates to nitrites, the formation of

peptone from albumins, the formation of kreatinin, and the change of hemoglobin into methemoglobin.

Chromogenic Bacteria.—There are a number of both pathogenic and numerous non-pathogenic bacteria which in their growth under certain conditions form pigments of various colors. One of the conditions necessary to pigment formation is the presence of magnesium and sulphur compounds in the culture medium. Free oxygen is also generally required. There are examples when free oxygen must not be present, as in the case of the Spirillum rubrum, which forms a red pigment only under anaërobic conditions. As a rule, the pigment is formed better at lower temperatures than are present in the incubator, and better in the dark than in the direct or diffuse sunlight. Some bacteria form a pigment only on certain culture soils, as, for example, the glanders bacillus on potatoes. Some chromogenic bacteria and the colors which they produce are:

The Staphylococcus pyogenes aureus, a golden-yellow pigment.

The Staphylococcus pyogenes citreus, a lemon-yellow pigment.

The anthrax bacillus, a brown pigment.

The glanders bacillus (on potato), a red-brown to yellowish-red pigment.

The Bacillus pyocyaneus, a green pigment.

The Bacillus violaceus, a violet pigment.

The Bacillus of avian tuberculosis, a yellowish-red to brown pigment.

The Bacillus prodigiosus, a red pigment.

The Bacillus cyanogenus (in milk), a blue pigment.

Some bacteria, particularly those occurring in sea water, form a fluorescent or phosphorescent material. The luminosity or phosphorescence of the ocean is due to the presence of these in enormous numbers.

Fermentation and Enzymes.—Bacteria and other low vegetable microörganisms, such as saccharomyces (yeast cells) and moulds, play an extensive role in the outside world as the cause of fermentative processes of organic compounds. Quite a number of soluble ferments or enzymes are furnished by pathogenic bacteria, and they lead to certain manifestations both in the bodies of infected animals and in artificial culture media.

The enzymes thus secreted are: *diastase,* the enzyme which splits up starch and forms maltose (malt sugar); *invertase,* which changes saccharose (a disaccharid) into glucose or grape sugar or dextrose (a monosaccharid); *rennet,* which precipitates the soluble casein from milk; *urase,* which decomposes urea; *lipase,* which splits the fats into their component fatty acids and glycerin; and a *peptonizing ferment,* which dissolves proteids and forms, from their complicated body, peptone and other simpler compounds. The action of the peptonizing ferment or enzyme can be well studied in artificial cultures prepared from blood serum or gelatin. As the peptonizing of these

substances goes on they become gradually liquefied. The peptonizing and liquefying property of certain pathogenic bacteria is very important from a diagnostic standpoint, and we therefore divide pathogenic microörganisms into two groups, *peptonizing* or *liquefying* and *non-liquefying* bacteria.

Other fermentative products which are formed by certain enzymes of pathogenic bacteria (colon bacillus) in the splitting of glucose are hydrogen and carbon dioxide; also occasionally alcohol and propionic acid. The anthrax bacillus when grown in the presence of sugar (glucose) forms lactic, formic, acetic, and sometimes succinic acid. The bacillus of malignant edema, under anaërobic conditions, decomposes glucose into ethyl alcohol, formic, butyric, and lactic acid. The Bacillus lactis aërogenes which occurs in the intestinal tract of man and the domestic animals forms lactic acid as its main product from sugar. Some pathogenic bacteria bring about putrefactive changes in milk and other albuminous materials. When milk is in the intestinal tract, however, these changes do not take place due to the presence of growing colon and lactic aërogenes bacilli.

Change of Reaction in Culture Soil.—Bacteria in general and pathogenic bacteria in particular require, as has been pointed out above, a certain delicate reaction of the medium in which they grow.

As a rule, the medium must be either neutral or slightly alkaline; more rarely slightly acid. Excesses in these slight degrees of acidity or alkalinity are fatal. Many pathogenic bacteria growing in culture media change their reaction considerably, and this change, particularly in the presence of certain substances, may be so great that the bacterium becomes weak or even dies. This is particularly true when sugar or glycerin are present. Many pathogenic bacteria form acids from these substances which may increase the acidity of the medium to such a degree as to destroy the bacterial life. Some bacteria, for instance the diphtheria bacillus, first increase the acidity of the medium and then reverse their action, neutralizing the culture soil, and finally make it alkaline to a certain degree. When the reaction of a culture medium is changed, other changes may occur depending upon the change of reaction. For instance, alkaline milk may be made acid by a growth of colon bacilli and the casein precipitated in the course of several days. This change is an important characteristic of some bacteria growing in milk, and is valuable as one of the means of identification and diagnosis.

Symbiosis.—It is found that some microörganisms when growing together in an artificial culture medium assist each other materially in their growth, or one may assist the other receiving no benefit itself, but at the same time experiencing no damage or hindrance in its growth. When two bacteria assist each other in this manner we speak of a symbiosis. The following groups of bacilli are properly classed under this head: The anthrax bacillus and the streptococcus; the

Staphyloccus pyogenes aureus and the influenza bacillus; the diphtheria bacillus and the streptococcus. On the other hand, two different species of bacteria when growing together may show a mutual antagonism and a retarding influence. Such antagonism exists between the anthrax bacillus and the Staphylococcus pyogenes aureus, the anthrax and typhoid bacilli. Other bacilli may not show any influence at all upon each other's growth as the typhoid and colon bacilli or as the cholera spirillum and certain non-pathogenic water spirilla. When symbiotic bacteria invade man or domestic animals simultaneously or nearly so, they are liable to produce a very virulent form of mixed infection. This has been observed in man in diphtheria with simultaneous streptococcus infection of the tonsils.

Influences Inimical to Bacterial Growth and Life.—We have already seen that bacteria for their metabolism, growth, and multiplication require certain definite conditions of the nutritive material, its concentration, moisture, reaction, the prevalence of a certain temperature, and the presence or absence of oxygen. It has also been shown that any excess of alkalinity or acidity is very detrimental, and that particularly mineral acids, even in very moderate concentration, speedily kill pathogenic bacteria. It has also been explained how their own accumulating metabolic products bring about the same result. In addition, the following outside inimical influences may be mentioned briefly.

Sunlight.—Many bacteria can stand the sunlight quite well; others, particularly pathogenic bacteria, are rapidly killed by such exposure; with them even diffuse daylight, when acting long enough, frequently proves fatal.

Electricity.—Electricity passing through a fluid culture medium forms *acids* and *alkalies* which are very detrimental to bacteria; *x*-rays, as far as known, have no effect.

Chemicals.—Some chemicals, such as corrosive sublimate, chloride of lime, permanganate of potash, carbolic acid, creosote, creolin, lysol, formalin, etc., even in weak concentration, have a tendency to kill bacteria rapidly. Chemicals which possess this property are called *antiseptics* or germicides.

Heat.—Heat, particularly when *moist*, is very inimical to microörganisms. As a rule, most disease-producing bacteria in the vegetative form are killed by a short exposure (ten minutes) to a comparatively moderate temperature (say 55° to 60° C.). This heat, however, must be moist; if dry and the bacteria are in the same condition they can often stand much higher temperatures and longer exposures. Spores, however, can often stand a long exposure to moist heat at high temperatures (100° C.). When instruments, sutures, and bandaging material are exposed to the heat of boiling water or steam at 100° C., it is done with the object of killing all bacteria and their spores. Such a procedure, provided it has been done successfully, is called *sterilization*.

Asepsis.—The term *aseptic* refers to methods and manipulations which protect a *sterile* or *relatively sterile* material from any further contamination or admixture with microörganisms. If, for instance, we shave the skin of an animal, wash and scrub it thoroughly with an antiseptic solution, then wash our hands in an antiseptic solution, and finally draw some blood from a vein with a perfectly sterile hypodermic syringe, we can say that we have drawn the blood in an *aseptic manner*. If everything has been done correctly, we have obtained the blood without contaminating it with microörganisms. If there are any found at all they must have been in the circulating blood as infecting microörganisms.

QUESTIONS.

1. At what range of temperature can bacteria as a class exist, grow, and multiply?
2. What bacteria are most limited as to the range of temperature at which they can multiply, and why?
3. What is the optimum temperature of growth of a bacterium?
4. What are the minimum and maximum temperatures of growth?
5. What is the optimum temperature for the (*a*) mammalian, (*b*) avian tubercle bacilli?
6. What are the maximum and minimum temperatures for these bacilli?
7. What range of temperature of growth do facultative parasites generally have, and why?
8. What is a thermophile bacterium?
9. Where do they occur and where do they find the conditions necessary for their multiplication?
10. What are thermotolerant bacteria?
11. What is meant by the thermal death point of a bacterium?
12. What do bacteria require for their nutrition? Can they grow without moisture?
13. What is the effect of desiccation upon bacteria and their spores?
14. What is the effect of desiccation upon
    (*a*) The spores of anthrax and tetanus bacilli?
    (*b*) The tubercle bacillus?
    (*c*) The glanders bacillus?
15. What is meant by the latent life of a bacterium or its spore?
16. From what materials can pathogenic bacteria best derive their food?
17. Name some pathogenic bacteria difficult to grow on artificial culture media.
18. Name some which have never been grown successfully.
19. What effect has too great a concentration of solids in an artificial culture medium upon the growth of bacteria?
20. What effect has the reaction of the culture medium upon the growth of bacteria?
21. What natural unchanged materials do certain pathogenic bacteria require before they will grow?
22. Explain the terms plasmolysis and plasmoptysis.
23. Explain the following terms:

Obligate anaërobe.	Facultative anaërobe.
Obligate aërobe.	Facultative aërobe.

24. Name some pathogenic anaërobes.
25. Name some pathogenic aërobes.
26. Name some pathogenic facultative aërobes.
27. How can strict or obligate anaërobes grow in the presence of oxygen?
28. What are some of the common metabolic products of bacterial life?
29. Name a bacterium which in the presence of peptone forms indol. What is indol?
30. What is meant when certain bacteria are said to have a reducing action?

31. What is meant when certain bacteria are said to reduce nitrates to nitrites?
32. What is a chromogenic bacterium?
33. What are some of the conditions *necessary* to the formation of bacterial pigments?
34. What are some of the conditions *favorable* to the formation of bacterial pigments?
35. Enumerate several species of chromogenic bacteria and describe their pigments.
36. Where are phosphorescent bacteria found? What is meant by this term?
37. What different enzymes are furnished by various pathogenic bacteria?
38. Explain the action and name the fermentative products of the following enzymes:

(*a*) Diastase.
(*b*) Invertase.
(*c*) Rennet.
(*d*) Urase.
(*e*) Lipase.
(*f*) Pepsin or trypsin.

39. What is meant by the statement that a bacterium belongs to the (*a*) liquefying; (*b*) non-liquefying type?
40. What does the anthrax bacillus form when growing in a medium containing sugar?
41. What does the bacillus of malignant edema form under similar but anaërobic conditions?
42. What does the Bacillus lactis aërogenes form from sugar?
43. How does bacterial growth affect the reaction of the culture soil?
44. What occurs in slightly alkaline milk when the colon bacillus develops in it?
45. What is meant by symbiotic pathogenic bacteria?
46. What is meant by bacterial antagonism?
47. Name some symbiotic and some antagonistic pathogenic bacteria.
48. What generally is the effect of direct sunlight upon pathogenic bacteria?
49. What is the effect of an electric current passing through a fluid culture medium containing bacteria? Upon what does the effect depend?
50. What is the meaning of the term antiseptics? Name some of those most commonly employed.
51. What is the comparative effect of dry and moist heat upon bacteria and their spores?
52. What is the meaning of the terms: (*a*) sterilization; (*b*) aseptic?
53. How can blood be obtained from an animal in an aseptic manner?
54. What is the meaning of the term bacterial contamination?

CHAPTER V.

OCCURRENCE OF PATHOGENIC BACTERIA IN NATURE—ROUTES OF ENTRANCE IN INFECTION.

MANY pathogenic bacteria are only facultative, and not strict parasites. They are not only found in infected persons and animals, but also in the outside world, where as saprophytes they find all conditions necessary for their existence, growth, and multiplication. There are a number of bacteria of this type which have an extensive distribution in nature and only occasionally invade the organism of animals, to lead there a parasitic existence. Among these we may mention the pus-producing (pyogenic) staphylococci. They are truly *ubiquitous*, and are found in the air, soil, water, on the external surfaces of all animals, in the various parts of their gastro-intestinal and respiratory tracts, and on the outside surfaces of all objects in nature, whether animate or inanimate.

Such organisms as the tetanus bacillus, the bacillus of malignant edema, and the Bacillus enteritidis sporogenes are prevalent in the soil; the first one of the three also occurs extensively in the intestines of the horse as a harmless commensale. The ray fungus is found in nature on many grasses, and the anthrax bacillus on pastures, where it multiplies and leads a saprophytic existence.

Certain pathogenic bacteria are only found in the outside world in the neighborhood of infected persons and animals which are responsible for their dissemination. In this manner typhoid bacilli are sometimes transferred to the soil, and the spirilla of Asiatic cholera to stagnant waters or even rivers, notwithstanding the fact that rapidly flowing rivers generally rid themselves quickly of pathogenic bacteria through the effect of sunlight and by the aid of algæ which destroy them. The anthrax bacillus, while undoubtedly found in nature independent of anthrax-sick animals, is often much disseminated by the latter.

There are, on the other hand, certain strictly parasitic pathogenic bacteria only found in the neighborhood of sick persons or animals from which they may spread for a short distance. Tubercle bacilli and glanders bacilli belong to this type and cannot, as far as known, exist in the outside world. Their presence in it is directly or indirectly dependent upon beings suffering with their specific diseases.

Pathogenic bacteria in the outside world may exist in or on the food of man and animals. The hay harvested from anthrax-infected prairies and pastures will contain this bacillus and its spores, and

may spread the disease wherever the hay is taken. Milk from tubercular cows may contain the tubercle bacillus, and when used in the raw state may spread the disease to animals, particularly to young calves and hogs, and man. Pathogenic bacteria which are excreted with the feces, urine, and other discharges may contaminate the floors and walls of houses, barns, stables, and also the bedding, straw, hay, and manure, and may exist on or in these objects for a long time. In cadavers properly buried pathogenic bacteria do not remain very long, not over a few months at most.

Portals of Entrance for Pathogenic Bacteria.—Pathogenic bacteria, whether they exist in nature as saprophytes like the tetanus or anthrax bacilli, or are there temporarily like the tubercle and glanders bacilli, or are directly disseminated from a sick to a healthy living being, must always enter a susceptible animal through certain portals of entrance in order to find the proper conditions for invasion and subsequent growth and multiplication. Sometimes a pathogenic bacterium may gain entrance by several routes, and sometimes it can enter but by one. For example, as far as known, the tetanus bacillus never infects a horse through the respiratory tract by inhalation nor through the gastro-intestinal tract by ingestion. It must always, in order to produce its disease, enter through a wound. Likewise, the typhoid bacillus and the spirillum of Asiatic cholera can infect man only through the gastro-intestinal tract by ingestion. On the other hand, the anthrax bacillus may invade the tissues through any one of these three channels.

Skin.—The intact skin with its outer layers of cornified epithelial cells forms an almost perfect barrier against the invasion of pathogenic bacteria. However, slight breaks in the skin are quite common, and these form the portals of entrance for a host of wound infections by the pyogenic cocci, the tetanus, anthrax, malignant edema, black-leg, necrophorus bacilli, by the organism of botryomycosis. Sometimes an animal may even be infected through the intact skin, particularly if the pathogenic organisms are rubbed in. In this way guinea-pigs may be infected with the bacillus of bubonic plague. While open, and particularly small, deep-seated puncture wounds offer a very favorable means of entrance to many pathogenic bacteria; it has been found that a wound once covered with granulation tissue becomes impenetrable. Evidently the granulations with their numerous young connective tissue, vascular endothelial cells and polynuclear leukocytes form a barrier which cannot be broken through.

Mucous Membranes.—The mucous membranes of the body are much less resistant than the skin. Some pathogenic bacteria can invade certain animals even through an intact mucous membrane. For example, if glanders bacilli are placed on the conjunctiva of a guinea-pig this animal contracts glanders; if plague bacilli are placed on the conjunctiva of a rat, the latter will contract bubonic plague.

Some mucous membranes on or near the external surface of the body seem to possess a stronger power of resistance to certain bacteria than to others. The nasal mucosa, which is very frequently exposed to the tubercle bacillus, is rarely affected by it. Tuberculosis of the nasal mucosa is rare. On the other hand, the nasal mucosa of the horse cannot offer any resistance to the glanders bacillus by which it is so often invaded. Pathogenic bacteria frequently find a portal of entrance through the tonsils and the nasopharynx.

Respiratory Tract.—The mucous membranes of the nose and the nasopharynx are structures which belong to the respiratory tract. This leads to a consideration of this tract as a portal of entrance. Infections of this type are very common, and they occur in such diseases as tuberculosis, glanders, influenza, pneumonia, nasal catarrh of birds, and in strangles of horses, the latter caused by the Streptococcus equi. Sometimes anthrax and the pneumonic type of plague are also contracted by inhalation.

Gastro-intestinal Tract.—The infection is brought about by the ingestion of food and water, in numerous diseases of man and the domestic animals, such as typhoid, Asiatic cholera, anthrax, actinomycosis, hog erysipelas, fowl cholera, tuberculosis, bacilliary dysentery, plague in rats, various hemorrhagic septicemias. The particular point where these pathogenic microörganisms gain entrance into the tissues varies greatly. It may be the mucosa of the mouth, as in actinomycosis of cattle, or it may be the small intestine, as in typhoid fever and Asiatic cholera in man, or again it may be the large intestine, as in anthrax or tuberculosis.

Genital Tract.—The genital tract forms the portal of entrance for pathogenic bacteria in *sexual intercourse*. Infectious diseases developed in this manner are perhaps more prevalent in man (gonorrhea, soft chancre, syphilis), but there are also dieases of this type among the domestic animals. Tuberculosis of the testicles of the bull has given rise to tuberculosis in the vagina of the cow, but this is exceedingly rare. There are, however, other animal infections more commonly transmitted in sexual intercourse. In cows, infectious abortion due to the korynebacterium abortus infectiosi of *Bang* is spread by the bull from already infected to healthy cows. The latter after the abortion harbor and discharge the bacterium for a long time, often for years, and abort again and again, continuing the spread of the disease through the bull. The infectious abortion of mares, due to a bacillus first discovered by Ostertag, is likewise spread by sexual intercourse, and its entrance is made through the genital tract. Another bacterial disease which finds its portal of entrance in the genital organs in sexual intercourse is the infectious vaginal catarrh of cows (kolpitis granulosa infectiosa bovum), caused by a specific streptococcus discovered by Ostertag and Hecker. Dourine of horses also makes its entrance in a similar manner. This disease, however, is not a bacterial but a protozoan infection (a trypanosomiasis).

Cryptogenetic Origin.—Pathogenic bacteria may first invade the body through wounds, by inhalation, or by ingestion, and be taken up by the blood or lymph current. They may then be deposited in the lymph glands and later carried to other places, where they multiply considerably to produce their most important pathologic lesions and changes. This happens in glanders where the important lesions are found far from the original place of entrance, and it occurs also in such diseases as pyelonephritis bacillosa bovis and necrotic liver abscesses in cattle due to the Bacillus necrophorus. Pus-producing microörganisms which sometimes produce very insignificant lesions at the portal of entrance are carried on and in another place produce profound purulent lesions. This occurs in bone abscesses (osteomyelitis) and in abscesses of the ovary. In fact, in many cases of this kind all trace of the small portal of entrance is lost. This is known as an infection of cryptogenetic origin.

Auto-infection.—Pathogenic microörganisms may enter through the respiratory or gastro-intestinal tract, and may not produce an infection at all. They may simply remain on the surface of the mucous membranes without penetrating the tissues. In this manner they may remain latent for a time, and then, when circumstances favor them, due to the animal becoming debilitated by overwork, poor food, chilling, etc., break into the tissue, there multiply and produce their specific infectious disease. Sometimes bacteria like the colon bacillus which live in the gastro-intestinal tract as harmless commensales may acquire pathogenic properties, invade the wall of the intestine, the peritoneum, the liver, etc., and cause an infectious disease with all of its stages and features. Such an infection which comes from within, that is, one that has no well-established outside source, is called an auto-infection.

Placental Circulation.—Bacteria may enter the *embryo in utero* through the placental circulation. This is a comparatively rare occurrence (as far as bacterial infections are concerned), since the maternal and fetal circulations are separate, and intact villi will generally not permit the passage of bacteria from mother to offspring. However, calf embryos are frequently infected with tuberculosis from the mother through the placental circulation, and such infection in human syphilis is quite common.

Insects.—Biting insects and other arthropods are responsible for a particular kind of wound infection. Sometimes these insects which have previously fed on infected material may spread an ordinary wound infection. Flies which have fed on contaminated material or cadavers may also spread disease by subsequent feeding on material destined as food for man or domestic animals. While these occurrences are by no means rare, they are what may be called accidental in their nature. There are, however, some insects which act as intermediary hosts for certain pathogenic microörganisms, and, therefore, are par

excellence carriers of the infection. The most important examples of such intermediary hosts are:

Mosquitoes, carrying malaria from man to man, or from birds to birds.

Tsetse-flies, carrying trypanosomiasis from animal to animal or to man.

Fleas and lice, carrying bubonic plague from man to man or animal to animal, or from animals to man.

Ticks, carrying Texas fever from cattle to cattle.

Bed-bugs, carrying relapsing fever from man to man.

Summary.—It has now been shown that the routes of entrance into the bodies of susceptible animals for pathogenic bacteria are the following:

1. Through wounds of the skin or mucous membranes.
2. By inhalation.
3. By ingestion with food or drink.
4. By intimate contact, particularly by sexual intercourse.
5. Through the placental circulation.

Excretion of Pathogenic Bacteria.—This takes place in different ways according to the place of entrance and the localization of the bacteria. An infected wound may be open from the start and discharge, and disseminate the infecting bacteria; or an abscess may have been formed when the excretion of the bacteria will only take place after the abscess has broken and established direct or indirect communication with the outside world. In tubercular infections, glanders, pneumonia, influenza, and strangles, the specific pathogenic bacteria are discharged through the natural communicating openings of the upper respiratory tract, or the pathologic products of these diseases may be swallowed and the bacteria discharged with the feces. In anthrax many of the bacteria are discharged with the feces, the urine, and also the bloody, foamy fluid flowing from the nostrils. In typhoid fever, Asiatic cholera, fowl cholera, bacillary dysentery, swine erysipelas, the disease producers are voided in enormous numbers with the feces. The organisms causing infectious abortion and infectious vaginitis in cows are discharged with the vaginal secretion and also to some extent with the urine; the latter contains innumerable specific bacteria in bacillary pyelonephritis in cattle.

QUESTIONS.

1. Name some facultative parasites which are extensively found in the outside world and only occasionally as pathogenic parasites.
2. Name some obligate parasitic bacteria which are only found where they have been spread by infected persons or animals.
3. Where are the anthrax bacillus and the ray-fungus found so that they may spread the disease with fodder?
4. What are the portals of entrance through which pathogenic bacteria may invade the body of a susceptible animal?

5. Name a pathogenic bacterium which generally finds entrance by one definite route only.
6. Name some pathogenic bacteria which may find entrance through several routes.
7. How does the intact skin act toward pathogenic microörganisms? How mucous membranes?
8. Name some pathogenic bacteria which generally gain entrance into the body of an animal through wounds.
9. Can animals be infected through the intact skin or mucosa, and if so, by what microörganisms?
10. How does granulation tissue act toward pathogenic bacteria?
11. How does the nasal mucosa act toward pathogenic microörganisms?
12. How do the pharyngeal tonsils act in the same respect?
13. Name some microörganisms invading the body through the gastro-intestinal tract.
14. At what point of the gastro-intestinal tract may they enter into the tissues?
15. Name a disease common in cattle which generally makes its entrance through the mucosa of the mouth.
16. Name some diseases in animals generally contracted through sexual intercourse.
17. How can a bull transfer the bacillus of infectious abortion to a cow?
18. Do pathogenic microörganisms always produce their most important lesions at their place of entrance? If not, what may happen?
19. What is meant by a cryptogenetic infection or an infection of cryptogenetic origin?
20. What is meant by a latent infection?
21. What is meant by auto-infection?
22. Name a bacillus which ordinarily lives in the intestines of man and animals as a harmless commensale, but which may produce auto-infections.
23. How can bacteria enter the embryo through the fetal circulation?
24. Name some insects and allied animals which as intermediary hosts may spread infectious diseases.
25. How and where are pathogenic bacteria excreted?
26. How can tubercle bacilli from the lungs be excreted with the feces?
27. Name some diseases of cattle in which the pathogenic bacteria are voided with the urine or vaginal secretion.

CHAPTER VI.

INFECTION—PHAGOCYTOSIS—OPSONINS.

INFECTION.

When pathogenic bacteria enter an animal by one of the routes indicated, and multiply in the cavities, organs, or tissues, and by so doing produce disease, an *infection has taken place*, and the disease produced is called an *infectious disease*. When a disease of this type can be transferred directly from one animal to another it is called *contagious*. Tuberculosis and glanders, for instance, are contagious diseases; but actinomycosis, while infectious, is not contagious, because there is no proof that it is ever directly transferred from one animal to another; the infective and infecting agent, the actinomyces fungus, being always taken up with the fodder. Texas fever is an infectious disease, but it is not contagious. However, even purely infectious non-contagious diseases may be directly transferred from the parent to the offspring through the placental circulation.

Toxins.—The question arises, How do microörganisms produce disease? Is it by their mere presence? Occasionally bacteria may so multiply that they accumulate in the capillaries, where they may lead to bacterial thrombi or emboli; but this is very exceptional. As a rule, bacteria produce disease by forming substances highly poisonous to the host in which these parasites multiply. These substances are called *toxins*. When a venomous snake bites it is not the small wound which causes disease and death, but the *venom* which gets into the circulation. In the same way it is not the presence of bacteria in the tissues and juices, but the toxins which they manufacture in their physiologic processes that, as a rule, cause disease.

Types of Toxins.—We must distinguish between two types of toxins, the *extracellular* or *soluble toxins*, and the *intracellular* or *insoluble endotoxins*.

Some pathogenic bacteria, for instance the tetanus bacillus and the diphtheria bacillus, in their growth in artificial media form soluble extracellular toxins. These get into the fluids in which the bacilli grow and the latter can be removed by filtration, so that a fluid is obtained containing the soluble toxins only. On the other hand, if colon, typhoid, or anthrax bacilli, or cholera spirilla, are grown in fluid culture media no toxin will be found in the fluid. Finely divided or decomposed bacteria of this kind must be disintegrated before it is possible to obtain their intracellular, ordinarily

insoluble toxins. Likewise, in the body of an infected animal such bacteria are in all probability subjected to more or less disintegration when their intracellular toxins are set free and exert their harmful influence. When pathogenic bacteria invade the body of an animal it may happen that they multiply enormously in a very short time, as in the case of the anthrax bacillus. Again, they may multiply very moderately, but evidently produce either a large amount of poison or a very powerful soluble toxin. The tetanus bacillus is of this latter type. Very little is known of the real intrinsic chemical nature of most of the toxins, and they cannot be distinguished by chemical tests. Still, their dissimilarity is clearly shown by their different effects upon susceptible animals and by the production of different signs and symptoms which made it possible to distinguish many infectious diseases long before microörganisms were known.

Virulence.—Not only bacteria of different types, but also the same kind of bacteria at different times, in different countries, and under varying conditions, produce diseases which vary much in intensity and in percentage of mortality. Bacteria which produce a violent form of the disease with high mortality are called *virulent bacteria* (the condition is called *virulence* or *virulency*). Often an epidemic starts with a low degree of virulency, gains more and more up to a certain point, and then diminishes again.

The virulence of bacteria depends more or less upon their power to multiply rapidly in the infected animal or upon their ability to produce a large amount of very powerful toxins. Virulency, however, does not depend upon the pathogenic bacterium itself alone, but also upon properties of different individuals of the infected species of animal, this variable factor is known as *individual susceptibility*. If animals of the same species are all exposed to one and the same infectious disease, some may contract it in a very violent form, other in a light form, still others not at all. This variability may depend upon age, sex, special breed, color, robustness, weakness, good or bad state of nutrition, or upon factors not yet recognized.

Increase of Virulence.—When pathogenic bacteria are studied experimentally it is generally found that their *virulency can be increased* in the following manner: A number of animals are first inoculated with a moderate dose. A certain percentage get very sick and die. From one of the very sick animals the pathogenic bacterium is obtained and again a number of animals are inoculated. This time a larger percentage get very sick and die. If this procedure is continued a number of times an infective bacterium is obtained of very great virulency which will kill all, or at least, a very high percentage. Nature does the same thing when an epidemic starts beginning with a mild form of the disease and developing into a very fatal, virulent form. On the other hand, at certain seasons, a fatal epidemic leads successively to milder forms of the disease.

Lessening of Virulence.—The virulency of pathogenic bacteria can be lessened by a number of procedures, such as:

1. Inoculating animals which are only slightly susceptible and obtaining the bacteria from them.

2. Growing the bacteria artificially at temperatures slightly higher than the body temperature of susceptible animals; that is, generally higher than the optimum temperature of such bacteria.

3. Growing bacteria in the presence of small amounts of antiseptics.

4. Growing for a few generations, on artificial culture media. Certain pathogenic bacteria will lose a good deal of their virulency when cultivated on such a medium for some time. The pneumococcus (the cause of pneumonia) is an example.

Attenuation.—Whenever virulent bacteria, through natural or artificial means lose some of their virulency they are said to have become *attenuated* and the process is called attenuation.

Avirulence.—When a virulent bacterium loses all its virulency and becomes non-pathogenic, it is said to have become avirulent. Some such avirulent bacteria cannot be made virulent again, but there are others which can be made so by being introduced successively into very susceptible animals.

Mixed Infection.—Sometimes more than one species of bacteria infects an animal; this is known as a mixed infection. A symbiosis, previously defined, would come under this head, and such symbiotic associations may be very detrimental to the infected animal and lead to a very *virulent form of the disease.*

The tetanus bacillus, as already stated, is an anaërobic bacterium. If a wound becomes infected with it and an aërobic bacterium which will absorb the oxygen, the tetanus bacillus has a better chance to multiply and form more toxins, and a most virulent form of the disease will result. Streptococci and diphtheria bacilli; staphylococci and colon bacilli often unite in symbiotic association in producing *virulent mixed infections.*

Mortality.—All of the primary signs and symptoms and pathologic changes of infectious diseases, such as the loss of appetite, weakness, inability to work, prostration, elevation of body temperatures, degenerative changes in the organs, disturbances of circulation, etc., are due to the elaborated and absorbed toxins.

Infectious diseases may have a high or low mortality, but there is practically no infectious disease which in every case leads to death. On the contrary, the majority of all such cases end, fortunately, in recovery.

Protective Agencies.—There are a number of factors which protect the body against the invasion and multiplication of pathogenic organisms, and there are other factors which limit both the multiplication of bacteria and the amount of toxins formed in cases where the bacteria have gained entrance to the body of the sick animal. If

these factors had not been developed in the evolution of the higher animals the latter would at all times be exposed to the invasion of pathogenic bacteria, and toxin production in an infected animal would go on until death resulted.

In the first place, pathogenic bacteria cannot, as a rule, gain entrance into the tissues of the body through a perfect skin or mucous membrane. Animals constantly inhale and ingest pathogenic bacteria. However, as long as the mucosa of the respiratory and gastrointestinal tracts and the skin are covered by unbroken layers of healthy epithelium, pathogenic bacteria have generally no chance of penetrating into the tissues. When breaks do occur in the epithelium the bacteria, of course, enter the tissues, and to counteract this invasion the body of the higher animals possesses *a number of protective agencies which are always at work against the multiplication and the toxic effect of the invading microörganisms.* These protective agencies are here considered systematically and in detail.

PHAGOCYTOSIS.

Metchnikoff was the originator and is today the strongest and most ardent exponent of the theory of *phagocytosis,* which owes its firm position today to the untiring efforts of its originator and his pupils.

Description.—The word *phagocyte* (derived from two Greek roots, means an *eating* or *feeding cell*) and the word *phagocytosis* indicate the act by which small fragments of bacteria are incorporated into a phagocyte and therein subjected to *digestion* and *assimilation.* Metchnikoff first observed phagocytosis in low animal organisms of the class tunicata, called *actinia.* These possess a common body cavity, not yet differentiated into a thoracic and abdominal cavity, known as a celom. If food or small particles of any kind are introduced into the celom they float around unchanged until they come in contact with the epithelial cells lining the celom cavity. As soon as this occurs the epithelia send out protoplasmic processes, pseudopodia, which surround the small particles, until finally they become completely incorporated in the cell protoplasm. If suitable they are then digested; if not, they are expelled. This is the process of phagocytosis as first observed by Metchnikoff.

The question then arose, Does anything like this process exist in higher animals? It was quite easy to show this to be the case. If the blood of a goose is defibrinated by whipping it with a bundle of pieces of wood or wire there is obtained a mixture of blood corpuscles and serum, minus the fibrin removed by whipping. This defibrinated blood can be mixed with physiologic salt solution, centrifuged, and the clear supernatant fluid pipetted off. If this is done several times there is obtained what is called in experiments of

this kind, *washed red blood corpuscles*. Now, if a few cubic centimeters of these washed corpuscles suspended in physiologic salt solution are injected into the peritoneal cavity of a guinea-pig and after a short time, say one hour, removed from the peritoneum together with an exudate from the abdominal cavity which has been mixed with them the following is found: The exudate contains numerous large mononuclear white blood corpuscles of the guinea-pig, and these cells contain many red bood corpuscles of the goose. If some of the peritoneal exudate is removed afterward at intervals of thirty minutes, the goose corpuscles will be found more and more digested in the large mononuclear cells of the guinea-pig, demonstrating that certain cells of mammalian animals possess phagocytic properties.

In 1883 Metchnikoff first claimed that phagocytosis protects the higher animals against infection by disease-producing pathogenic bacteria. His opponents, however, showed that in animals dead from anthrax, numerous anthrax bacilli were seen in the blood, none of which were being taken up or digested by phagocytic cells. Metchnikoff then succeeded in demonstrating that in animals not susceptible to anthrax such phagocytosis of bacilli takes place and that the lack of susceptibility depends upon the fact that the anthrax bacilli are taken up and destroyed by phagocytes.

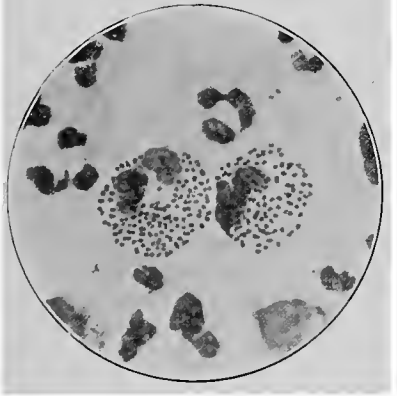
Phagocytic Cells (Macrophages and Microphages).—It may very properly be asked, What kind of cells of the higher animals possess the power of phagocytosis? With a single exception all cells which have the power to act as phagocytes are derived from the middle germinal layer, the mesoderm or mesoblast. Among these cells two classes are represented, namely, wandering cells or leukocytes and fixed connective-tissue cells. Not every white blood corpuscle or leukocyte can act as a phagocyte. As a rule, only some of the polynuclears and the large mononuclears are phagocytic. Among the fixed tissue cells endowed with the power of phagocytosis are the large mononuclear cells of the spleen, the lymph sinuses and the lymphatic endothelia of the lymph clefts, the bone corpuscles, the myeloplaxes of the bone marrow, and other giant cells. Metchnikoff divides all the phagocytic cells into macrophages and microphages (large and small phagocytes). The former destroy particularly dead or foreign cells (foreign blood corpuscles) while the latter generally destroy bacteria. (The largest kind of macrophages, the multinuclear giant cells, also destroy bacteria.) In general it can be stated that the wandering cells, the leukocytes, are more important in phagocytosis than the fixed cells.

Experimental Demonstration of Phagocytosis.—Phagocytosis of pathogenic bacteria can be easily demonstrated by numerous experiments. If a small amount of a twenty-four-hours-old culture of virulent anthrax bacilli is injected into the lymph sac of a frog, and if from time to time some of the injected fluid is removed by the aid of a small pointed glass pipette, it will be found that the white corpuscles of the frog take up and digest more and more anthrax bacilli until

the latter have entirely disappeared. It can also be shown that the anthrax bacilli which the frog's leukocytes take up are first alive and virulent and remain so until the process of intracellular digestion and destruction has progressed to a certain point.

A similar experiment can be made by injecting a bouillon culture of anthrax bacilli into a chicken. Here, again, the leukocytes of the chicken act as phagocytes and destroy the anthrax bacilli. However, if the temperature of the chicken is reduced by immersing its feet in cold water, or by administering to it large doses of chloral or antipyrin, the leukocytes will be so damaged that they lose their phagocytic power. They do not now take up and destroy the bacilli; these multiply and the chicken dies from anthrax. It can be shown that the lack of susceptibility of pigeons to human tuberculosis is due to the power of that bird's leukocytes to destroy tubercle bacilli of human derivation.

FIG. 24



Numerous diplococci (gonococci) inside of the protoplasm of two polynuclear leukocytes (phagocytes). × 1000. (Author's preparation.)

Guinea-pigs are ordinarily not susceptible to an infection with the Streptococcus pyogenes. If cultures are injected into the peritoneal cavity of the guinea-pig the cocci are taken up and destroyed by phagocytes. However, very virulent cultures of streptococci frequently kill guinea-pigs, and it can be shown that such very virulent cocci are not destroyed by phagocytosis. Rabbits are very susceptible to the tetanus toxin, but if washed tetanus spores free from toxin are injected into the peritoneal cavity, phagocytes take up these spores and make them innocuous.

Immunity.—All of these examples show that animals without receiving any preliminary treatment can protect themselves against invasion and multiplication of pathogenic bacteria by phagocytosis. Animals may be very susceptible, however, to certain pathogenic bacteria because phagocytosis does not naturally occur, and, in consequence, a *natural immunity* does not exist. Fortunately in these cases an *artificial immunity* can generally be brought about.

It is well, at this point, to understand that we mean by immunity the power of an animal to resist the invasion of pathogenic bacteria (see below). This power may be brought about artificially by injecting into a susceptible animal less than a fatal dose of a pathogenic bacterium or a weakened, that is, an attenuated, bacterium or a weakened virus or vaccine.

If, for instance, such an attenuated strain of the anthrax bacillus (anthrax vaccine) is injected into a susceptible animal, its leukocytes, heretofore non-phagocytic with reference to anthrax bacilli, are endowed with phagocytic properties.

Stages.—There are distinguished in phagocytosis three different stages—namely, the *approach* of the phagocyte to the bacterium; *inclusion*, and finally, *digestion*, or destruction, of the microörganisms.

Chemotaxis.—It is one of the most remarkable facts that phagocytes possess a *bacteriochemical sensibility*. This term means that phagocytes are attracted toward certain bacteria, as iron filings are attracted to a magnet. It is easy to demonstrate this and to show how phagocytes can even wander through a vessel wall toward a place where certain bacteria have been injected. This peculiar attraction which acts over a distance is known as a positive chemotaxis or chemiotaxis. It can, on the other hand, be shown that some bacteria have a repelling influence toward phagocytes which causes them to flee. This repelling power is called *negative chemotaxis*. When bacteria exert neither an attracting nor a repelling influence upon polynuclear leukocytes, we speak of an *indifferent chemotaxis*.

Aggressins.—It is a very interesting fact that the most virulent strains of certain bacteria, for instance, very virulent streptococci or pneumococci, exert a repelling influence toward the wandering leukocytes, and in this manner prevent phagocytosis. It is very probable that the power of such virulent strains of pathogenic bacteria depends upon certain of their secretory products, and these have been called aggressins.

Phagocytosis and Spreading of Disease.—In considering the process of phagocytosis as one of the protecting agencies of the body against the invasion and multiplication of pathogenic bacteria, it is important and necessary to remember that phagocytes often swallow more bacteria than they can digest. These bacteria remain alive, secrete their toxins, weaken, and finally kill the leukocytes. Death of the latter may not occur for some time, and meanwhile they may wander away after having engulfed several bacteria, and thus transport infecting bacteria to a place distant from their first point of entrance. In other words, under some conditions phagocytes, instead of acting for good, as is generally the case, may do harm by spreading infection from one place to another.

Preparation of Serum-free Leukocytes.—Leukocytes of man or animals can be prepared in such a manner that they are free from every trace of blood serum. This is done in the following manner: About fifteen to twenty drops of blood are drawn by puncturing a finger or an ear with a sterile surgical needle and the blood is allowed to fall directly into a glass centrifuge tube which contains a $1\frac{1}{2}$ *per cent. watery solution of sodium citrate.* This solution prevents the coagulation of blood. Blood and citrate solution are mixed by shaking and the mixture is then centrifuged until a clear upper stratum is

formed. This is drawn off with a pipette and the centrifuge tube is now filled by pouring into it a 0.85 per cent. (physiologic) salt solution. The tube is shaken so that the sediment is thoroughly mixed with the salt solution and again centrifuged. The clear upper stratum is once more drawn off and the sediment is once again shaken with the salt solution. After a final centrifuging the tube will present three layers: a lowest scarlet sediment of red blood corpuscles, a narrow grayish-red ring which contains the leukocytes, and a perfectly clear top layer which is the salt solution. The latter is very carefully drawn off with a pipette with rubber bulb, so that the gray ring of leukocyte is not disturbed. After the salt solution has been drawn off the grayish layer is carefully drawn into a small pipette and at once expelled into a watch-glass, which is covered to prevent evaporation. The watch-glass now contains a mixture composed of salt solution, suspended leukocytes, and also a few red blood corpuscles. Their presence, however, does not interfere with the use of the suspension or emulsion of washed leukocytes. When these are to be employed either in experimental work or in order to ascertain the opsonic index (see below), a small amount of the suspension is drawn into a fine glass pipette. If a little air bubble is now drawn up and then an amount of bacterial emulsion, equal to the leukocyte emulsion, these two fluids can be mixed in the pipette by repeatedly drawing them up and expelling them on a clean slide. After they have been finally drawn into the pipette the point of the latter is sealed in a flame, and the pipette and its contents can now be placed in an ordinary bacterial incubator or into a so-called *opsonic incubator*. The mixture is generally left in the incubator for one-half hour; then the pipette is removed, its sealed point broken off, and the contents blown onto a clean slide. The drop is then spread with the margin of another clean slide, the preparation is air dried, fixed in the flame or in alcohol, and stained. It can now be examined with the oil-immersion lens of the microscope like any other bacterial preparation. If the test has been made as described above, it will be found on microscopic examination that very little if any phagocytosis has occurred.

Opsonins.—It has been found by Wright and Douglass that the blood serum contains certain substances which so prepare bacteria that they are subsequently taken up by phagocytes with avidity. These substances contained in the blood serum have been called opsonins (a word derived from a Greek verb which means to prepare).

Characteristics.—It is not known definitely what these substances are. Some investigators strongly maintain that they are identical with hemolytic and bacteriolytic amboceptors (see below). Other serum investigators claim that they are bodies, *sui generis*, and not identical with any of the other antibodies. Be this as it may, it has been shown beyond a doubt that the blood serum contains certain

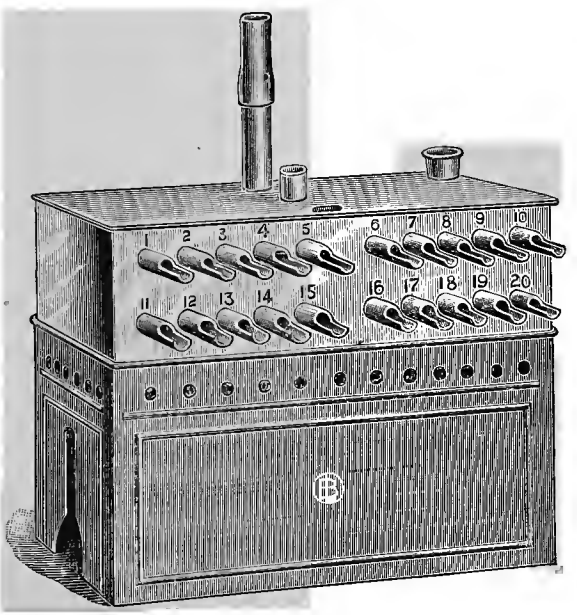
substances which prepare bacteria for subsequent phagocytosis. Whether these substances are originally formed in the blood serum, or whether they are furnished by decomposing leukocytes, as Metchnikoff and his followers claim, is likewise not settled.

FIG. 25

Opsonizing pipette into which serum, washed leukocytes, and a bacterial emulsion have been drawn up. (Miller in Therapeutic Gazette.)

Experimental Demonstration of Opsonins.—The presence of these substances in the blood serum can be shown by the following experiments. In one test leukocytes washed in physiologic salt solution and a freshly prepared bacterial emulsion are mixed in equal proportions and placed in the incubator for one-half hour. In a simultaneous test, washed leukocytes are mixed with blood serum and a freshly prepared bacterial emulsion, and the mixture is treated as above. After one-half hour, microscopic preparations are made from both mixtures. It will be found that in the mixture where blood serum was lacking phagocytosis has not taken place, while in the other test where blood serum was present a good deal of phagocytosis has occurred. This clearly shows the influence of the opsonins of the blood serum.

FIG. 26



Opsonic incubator.

Hektoen has shown that anthrax bacilli are taken up by the leukocytes of the dog in the presence of dog's blood serum. If the leukocytes are washed several times with physiologic salt solution, so that they are entirely free from serum, and then mixed with a bacterial emulsion, no bacteria are taken up by phagocytosis. Hektoen *also* demonstrated that the opsonins in the dog's blood serum which bring about the phagocytosis of anthrax bacilli are destroyed if the serum is subjected to a temperature of 56° to 60° C. for thirty minutes. The leukocytes themselves are made unfit for phagocytosis if heated for thirty minutes at 45° C., but these cells of the dog contain a thermostabile anthracidal substance, not destroyed by heating to 56° C., which can be extracted with distilled water after self-digestion. In working with anthrax bacilli to determine phagocytosis it is sometimes difficult, on account of the length of the pseudofilaments, to determine whether a given leukocyte is or is not engaged in phagocytosis. This difficulty can be easily overcome by systematic comparisons of preparations from experiments with or without dog's blood serum.

Opsonic Index.—When the blood serum of several healthy persons or animals is examined with reference to the same pathogenic microorganism, it is found that the amount of opsonin present gives a fairly constant average. The amount of the opsonin, of course, cannot be estimated directly. Indirectly it is calculated by the amount of phagocytosis which occurs in properly arranged, simultaneous, and equivalent tests. *It can also be shown that, as a rule, in many chronic bacterial infections the amount of opsonin in the blood serum falls below the normal.* For instance, it will be found that one hundred cattle leukocytes with the mixed blood sera from five healthy cattle will take up by phagocytosis two hundred tubercle bacilli; while in the same experiment with sera from five cases of mild chronic tuberculosis, each serum being used separately, the number of bacilli taken up will be from 120 to 150. It is customary to compare the number of bacilli taken up in the test with the mixed sera from the healthy animals or persons with the number taken up in the test with a serum from an infected animal, and the figure obtained by dividing the latter by the former is called the opsonic index of the blood. In the example above the opsonic index of the sick animals would be $\frac{120}{200}$, or $\frac{130}{200}$, or $\frac{150}{200}$; that is, 0.6 to 0.75. *As a rule, the opsonic index in chronic bacterial infections is always low; that is below* 1 (one being the normal standard).

Vaccines.—It has been shown experimentally that the opsonic index for a pathogenic bacterium can be raised by injecting into the infected person or animal a small dose of a vaccine or bacterine prepared from the particular bacterium which causes the infection.

Vaccines are generally prepared by obtaining first the bacterium which causes the chronic infection in pure culture, and then heating the *bacterial emulsion* thus obtained to a temperature which will just kill the microörganisms without damaging them too severely. Such

a *vaccine* or *bacterine* when injected into a person or animal suffering from the particular chronic infection and having a low opsonic index, will, during the first twenty-four hours, generally *lower the index a little more.* This is called the *negative phase.* After the first twenty-four hours, however, the index will rise from day to day, until after the sixth day it is generally considerably above the normal opsonic index. This is called the *positive phase.* Then the index begins to drop again. If, now, on the sixth or seventh day, another vaccine injection is given, the index instead of going below normal will rise again considerably above normal. It has been found that, very frequently, simultaneously with the rise of the opsonic index the general condition of the sick person or animal improves and complete recovery may take place. This vaccine, or, as it is also called, *bacterine treatment,* under guidance of the opsonic index, which has to be ascertained from day to day, is an exceedingly laborious, time-consuming, and delicate task, but it has been tried in man in thousands of cases. It has been found to bring favorable results in chronic local infections of the tubercle bacillus, the staphylococcus, streptococcus, pneumococcus, gonococcus, colon bacillus, etc.

The treatment is not applicable in acute violent infections and in very advanced generalized conditions, as in chronic tuberculosis, acute septicemia, pyemia, pneumonia, etc.

Details of Method of Obtaining the Opsonic Index.—The details of the procedure to obtain the opsonic index and to prepare the bacterine or vaccine in the case of a horse suffering from a local chronic staphylococcus infection are as follows: Ascertain by microscopic examination and cultures that the Staphylococcus pyogenes aureus (the common pus-producing staphylococcus) is the cause of the chronic suppuration. The steps now necessary to obtain the opsonic index of the horse's blood (for the Staphylococcus pyogenes aureus) are as follows:

1. Prepare a culture of the infecting microbes, and grow in the incubator for eighteen to twenty-four hours. Shake well if in bouillon, or if on agar make a suspension in salt solution, then heat in a water bath for one-half hour at 55° C. Shake well again, centrifuge, and pipette off some of the supernatant uniformly cloudy fluid which is a homogeneous emulsion of the Staphylococcus pyogenes aureus.

2. Get some blood from a healthy horse, allow it to fall into a centrifuge tube containing a 1.5 per cent. solution of citrate of sodium. This fluid will prevent coagulation. Mix well by shaking. Wash as described before in physiologic salt solution; finally, centrifuge well once more. Three layers are formed: the lowest layer of red blood corpuscles, a clear upper fluid layer, and between them a thin grayish red film. The latter contains the white corpuscles. Pipette off the clear fluid. Now, pipette off the leukocytes. They will be mixed with some red corpuscles, but this is of no significance.

3. Get blood from three healthy horses. Separate the serum from the corpuscles in each case by centrifuging or coagulation. Mix the three sera in equal proportions. This mixture, in work with opsonins, is called the *pool.*

4. Get blood from the sick horse, and after coagulation, separate the serum from the corpuscles.

FIG. 27

After the pipette has been prepared, its contents are repeatedly expelled and drawn up again in order to mix the three constituents well. (Miller.)

FIG. 28

After the pipette has been in the incubator, the contents are blown on a slide and the drop is drawn out. (Miller.)

5. Mix in equal amounts the washed white blood corpuscle emulsion with the three-serum mixture from healthy horses (the pool), and with the centrifuged-heated culture (emulsion) of the Staphylococcus pyogenes aureus.

6. Mix in another small pipette equal amounts of the white blood corpuscle emulsion of the serum of the sick horse and of the centrifuged heated bouillon culture of the Staphylococcus pyogenes aureus.

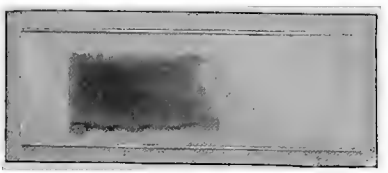
7. Now place pipettes No. 1 (three healthy horses' serum mixture—the pool) and No. 2 (serum from sick horse) in the incubator for one-half hour.

8. Make a smear preparation from pipette No. 1 and also from No. 2. Dry, fix, and stain.

9. Count 200 leukocytes in specimen No. 1, and count how many cocci have been taken up by phagocytosis in the 200 leukocytes.

10. Do the same with No. 2.

FIG. 29

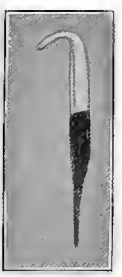


The smear, ready to be air-dried, fixed and stained. (Miller.

The result, for example, may be as follows: 200 leukocytes in No. 1 have taken up 300 cocci (this is the mixture of the sera of three normal horses); 200 leukocytes in No. 2 (sick horse's blood serum) have taken up 150 cocci; therefore, taking the normal opsonic index as 1 (one), the low opsonic index of the serum of the sick horse is 0.5.

When it is found that the opsonic index is low, vaccine treatment (in our case the Staphylococcus pyogenes aureus vaccine) is indicated.

FIG. 30



Wright's blood capsule filled with blood and ready to be centrifugalized for separating the serum from the clot. (Miller.)

In the above steps, to determine the opsonic index, small U-shaped tubes, or, according to Wright, peculiar glass capsules are used for collecting the blood sera. The mixing of the emulsion of leukocytes of the sera and the bacterial emulsion is done in small pipettes. The fluids are drawn up in equal amounts, first one, then a little air bubble, then the second, and so forth. The three different fluids (serum,

leukocytes, bacterial emulsion) are mixed by being several times drawn up and again expelled from the pipette into a watch crystal or glass slide. The mixtures in the little pipettes, after these have been sealed in the frame, are finally placed in the incubator or a special opsonic oven. After half an hour the mixtures are blown on a slide, spread out, air dried, stained, and then the bacteria taken in by 200 leukocytes are counted.

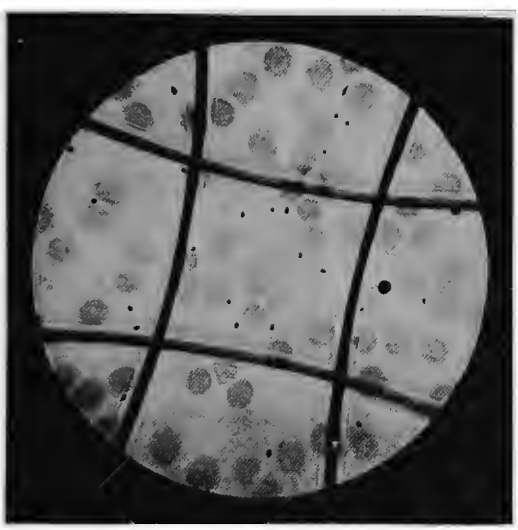
FIG. 31

Opsonic pipette after mixture of the three constituents; the tip has been fused and the pipette is ready for the opsonic incubator. (Miller.)

Preparation of Vaccines.—Considerable care has to be exercised in the preparation of the bacterial vaccines or bacterines used for therapeutic injections. Unless an *autovaccine*, that is, a vaccine, from the bacteria infecting the patient it wanted the vaccines are generally procured from one of the pharmaceutical houses which prepare them in their biological laboratories. The autovaccine must be prepared directly from the infecting organism. As a first step, a pure culture must be obtained, which may be raised in bouillon or on slanted agar; the latter is probably the better. The growth is then removed with a platinum loop or spatula, mixed with physiologic salt solution, shaken long and thoroughly, and then heated to kill the bacteria. The remaining coarser particles are removed by centrifuging and the uniform emulsion is *standardized by estimating the number of bacteria present per cubic centimeter*. This is done by collecting and mixing one part of the vaccine, three of physiologic salt solution, and one of normal human blood in a blood pipette. After these ingredients have been thoroughly mixed, drops are blown on a clean slide, spread, air dried, fixed, and stained. Then 200 red blood corpuscles are counted and the number of bacteria in the same number of fields is ascertained. Since it is known that each cubic millimeter of normal human blood contains five million erythrocytes, the number of bacteria present in the same amount of fluid can easily be calculated from the number counted in the same spaces which contained the 200 erythrocytes. After the calculation has been made the vaccine can be so diluted that it contains from 50,000,000 to 300,000,000 bacteria per cubic centimeter. These are the average doses used in the vaccine treatment in man. A small amount of lysol or some other antiseptic should be added to the vaccine, unless it is used at once. In counting the blood corpuscles and bacteria, it is advantageous for accurate work to have the microscopic field of vision limited by placing horsehairs in the form of a rectangle in the eyepiece or by the use of a square diaphragm.

The task of preparing vaccines and ascertaining the daily opsonic index is so delicate and time-consuming that it is out of the question for the practitioner to do the work himself. If he wants to treat cases of chronic bacterial infections by the vaccine or bacterine method, he must first ascertain what particular microörganism does the harm, and then get a vaccine prepared from the identical species and use it by hypodermic injection every sixth or seventh day. It is best to start with a minimum dose and watch its effect upon the animal treated. It should be remembered that general improvement frequently, but not always, is coincident with a rise in the opsonic index.

FIG. 32



Showing a microscopic field of the vaccine-dilute blood mixture, prepared in order to standardize the vaccine. (Miller.)

Archibald, in a paper read before the 1909 Chicago Meeting of the American Veterinary Association, and printed in the Transactions of the Association, stated that he had very good success with autogenic vaccines prepared by himself in the treatment of quittors, fistulæ, and other infective troubles.

Opsonins are *specific bodies*, which means that the injection of a staphylococcus vaccine will raise a low opsonic index for staphylococci but not a low index for tubercle bacilli or any other bacterium.

QUESTIONS.

1. What is an infectious disease?
2. What is a contagious disease?
3. Name some infectious diseases, also some contagious diseases.
4. How do microörganisms produce disease?
5. What is a toxin, a soluble toxin, and an intracellular or endotoxin?
6. Name some pathogenic bacteria which form soluble toxins. How can they be obtained free from bacteria?
7. Name some bacteria which form only insoluble endotoxins. How are the latter set free?
8. Name a pathogenic bacterium which multiplies very freely in the body and one which multiplies sparingly, but produces a powerful soluble toxin.
9. What is meant by a virulent bacterium and by virulency?
10. What is meant by individual susceptibility?
11. What is meant by attenuation?
12. What methods can be employed to attenuate virulent bacteria?
13. What is meant by an avirulent bacterium?
14. What is a mixed infection?
15. What is symbiosis?
16. Why does not an infectious disease attack every individual? Why does it not always kill?
17. What is a phagocyte? What is phagocytosis?
18. Who first studied phagocytosis, and in what animals?
19. What happens if we inject washed goose-blood corpuscles into the peritoneal cavity of a guinea-pig?
20. What is the procedure of washing mammalian or avian blood corpuscles?
21. What kind of cells of higher animals possess the power of phagocytosis?
22. What are macrophages? What are microphages? How do they differ as to their phagocytic properties?
23. How can the phagocytosis of anthrax bacilli be demonstrated?
24. How do very virulent streptococci act with reference to phagocytosis?
25. What is meant by immunity?
26. What is an attenuated virus or vaccine?
27. What are the three stages of phagocytosis?
28. What is positive, negative, and indifferent chemotaxis?
29. What is an opsonin?
30. What is the effect of washed leukocytes upon a bacterial emulsion? What is their effect in the presence of blood serum? Why is this effect produced?
31. Describe comparative experiments to show the effect of the absence or presence of serum upon phagocytosis.
32. Describe such a set of experiments with dog's leukocytes and anthrax bacilli.
33. Are opsonins present in normal healthy animals in very variable or in rather constant amounts?
34. How can the amount of opsonin present be estimated?
35. What about the amount of opsonin in chronic bacterial infections?
36. What is meant by the opsonic index? How is it estimated?
37. If in the mixture of healthy sera in one experiment 2001 eukocytes have taken up 250 bacteria, and in the experiment with the serum of the sick person or animal only 100 bacteria have been taken up by phagocytosis, what is the opsonic index of the latter serum?
38. What is the effect of vaccine or bacterine injection upon the opsonic index?
39. What is the negative phase after vaccine injection? What is the positive phase? How long does the latter generally last? What occurs then?
40. Describe in detail how to obtain the opsonic index in case of a chronic staphylococcus infection in a horse

CHAPTER VII.

ANTIBODIES—IMMUNITY—EHRLICH'S SIDE-CHAIN THEORY—THE WASSERMANN SERUM TEST.

ANTIBODIES.

Toxins and Antitoxins.—It has already been stated that some pathogenic bacteria secrete very powerful soluble toxins which enter the general circulation. Whenever such toxins circulate in the blood there is a tendency to the formation of bodies which neutralize them, and bring about a cure, provided that the toxins are not overabundant and have not already done irreparable damage. When tetanus toxins are in the system of a horse they are usually generated so quickly and abundantly that before they can be neutralized by natural or artificial means irreparable damage has been done to some part of the body, generally the central nervous system. The bodies which neutralize the *soluble toxins* are called *antitoxins*. Their effect can be best understood by comparison with the well-known chemical reaction between acids and alkalies. There is a disease common in man, and sometimes found in the domestic animals, which is characterized clinically by the secretion of sugar in the urine and by the inability of the body to properly split up and utilize this sugar. Consequently, sugar finds its way into the blood, and from this carbohydrate a large amount of organic acid is formed. No being could exist with a large amount of free acid in the blood, so the system at once corrects the defect by furnishing to the blood a large amount of ammonia to neutralize the organic acids. Somewhat similarly the antitoxins neutralize the toxins. Just as hydrochloric acid can be neutralized in the test-tube with ammonia, so can a soluble toxin be neutralized with its antitoxin. The principle is the same, although the process is a much more complicated one than the neutralization of an acid by an alkali. The toxin-antitoxin mixture can be injected into a susceptible animal without producing any ill effects. Thus the formation of antitoxins is another means by which the body protects itself against pathogenic bacteria; that is, against one of their most important products, the toxins.

Agglutinins, Lysins, Precipitins.—If cholera spirilla are injected into the peritoneal cavity of an animal which is not susceptible to them, and from time to time removed by the aid of small capillary pipettes, it will be noticed that the spirilla soon lose their motility, become glued to each other, forming small lumps, indistinct in out-

line, and are finally completely dissolved. These occurrences are called:

Immobilization (loss of motility).
Agglutination (becoming glued together).
Lysis (solution).

It can be shown that all of these processes favorable to the infected animal, but detrimental to the pathogenic infecting bacteria, are brought about by definite bodies contained in the blood serum and juices of the system. These substances are known as agglutinins and lysins. If human blood serum is injected repeatedly into the body of a rabbit at intervals of ten to fourteen days, and some time after the injection a little of the rabbit's blood serum is obtained, it will be found that a very small amount of the rabbit's blood serum added to a very dilute solution of human blood will cause a clouding or precipitation of exceedingly fine flocculi. Something has evidently been formed in the rabbit's blood serum which precipitates something from the human serum. The body or bodies so formed in the blood serum of one animal treated with the serum of another animal are called *precipitins*.

The specific test with the blood of a rabbit *sensitized* against human blood serum is a very important one from a medicolegal or forensic standpoint, because it makes possible to identify human blood stains in criminal cases and to differentiate them from the blood of any other animal. This test is much more delicate than measuring the erythrocytes in order to distinguish between human and other red blood corpuscles.

The three bodies, *agglutinins*, *lysins*, and *precipitins*, like *opsonins* and *antitoxins*, belong to the *protective substances* of the animal body against pathogenic bacteria. A common name for all of these bodies inimical to pathogenic bacteria is *antibodies*. Many antibodies are normally present in higher animals, but often only to a small extent and sometimes not at all. However, the system can be stimulated to manufacture antibodies when not present, or to increase enormously those present to a small extent, by the injection of either live or dead pathogenic bacteria. Such injections must be practised with certain precautions and with the observation of certain rules, otherwise instead of strengthening the animal against bacterial invasions, it will be killed. The effect of injecting live or dead bacteria into an animal is very different from injecting certain chemical poisons. An animal can be accustomed to stand successively increasing doses of morphine, strychnine, arsenic, etc., but in spite of such treatment, no antibodies to morphine, strychnine, or arsenic are formed. The antibodies formed when pathogenic bacteria are injected are *specific*, *i. e.*, when, for instance, tetanus bacilli are injected antibodies are formed against tetanus bacilli and their toxins, but they have no effect on diphtheria, glanders, or anthrax bacilli. If it is desired to form antibodies against a certain bacillus,

this same bacillus must always be injected. Antibodies are not only formed against bacteria, but against other organized material. If, for instance, human blood serum is injected into a rabbit, there will be formed antibodies against human serum in the rabbit's blood serum, and if sheep's blood corpuscles are injected into a rabbit there will be formed in the latter's blood serum antibodies against sheep's blood corpuscles, etc.

Antigens.—Any body, be it a bacterium, an animal, or a vegetable cell or other organic product of any kind, that is injected into an animal by the paraenteral[1] route and causes the formation of antibodies is called an antigen (meaning to produce antibodies).

Vaccines and Antitoxic Sera.—The term vaccine (also vaccination) is derived from the Latin word *vacca* (cow). It was first used for the procedure of inoculating superficially the arm of a person with the contents of a cowpox vesicle or the contents of the pustule from another person. This method, as is well known, was and is used to protect persons so vaccinated against smallpox. Today the term vaccine is used in a general sense for any infecting living virus (bacterium, protozoan, or invisible infectious microörganisms, or their toxins), either in full strength (virulency), in a very small does, or in an attenuated form in a larger dose, for the purposes of producing antibodies; that is, for the purpose of preventing or curing disease. Some pathogenic microörganisms may even be killed by high degrees of heat and still be effective as vaccines in the production of antibodies.

Preparation.—Reference has been made to methods by which pathogenic bacteria can be attenuated in order to be used in the preparation of vaccines. These various methods are:

1. Temperatures moderately higher than the optimum—anthrax vaccine.
2. Temperatures which will kill the bacteria—tuberculin, black-leg vaccine, staphylococcus vaccine, streptococcus vaccine, pneumococcus vaccine, colon bacillus vaccine, plague bacillus vaccine.
3. The addition of antiseptics in small amounts—carbolic acid, for anthrax vaccine; iodine, for tetanus vaccine.
4. Drying—rabies virus.
5. Digesting the bacterium—cholera spirillum vaccine.
6. Prolonged cultivation in artificial media—fowl cholera vaccine.

Vaccines are, as seen above, generally prepared artificially outside of the animal body. Antitoxins, however, can only be prepared in the body of an animal. The principle of the procedure is generally as follows: Inject into an animal, say a horse, successively increasing doses of, for example, a diphtheria toxin, and finally larger amounts of cultures of virulent diphtheria bacilli themselves. These

[1] A paraenteral method is one of incorporation of a substance into an animal subcutaneously, intraperitoneally, subdurally, intravenously, or by any other route than the gastro-intestinal tract.

injections have to be made at suitable intervals and a new one must not be made before the effects of the previous one (prostration, fever, etc.) have entirely disappeared. By this method there is manufactured in the body of the animal, *i. e.*, in its blood serum, an enormous amount of antitoxin. After the proper prolonged treatment the blood can be drawn off and the serum separated from the clot (everything thing should be done, of course, under aseptic precautions).

Antitoxic Units.—The serum so obtained is called an antitoxin, an antitoxic, or an immune serum. It is necessary to ascertain the value of an antitoxic serum. This is done in animal experiments in which both toxins and the antitoxic serum to be tested are used. The animals employed in the experiments are generally guinea-pigs. The *unit measure* of an antitoxin generally employed is that amount which will protect a guinea-pig of about one-half pound weight against *ten times the ordinary fatal dose.* For instance, it is found that 0.01 c.c. of a strong diphtheria toxin will kill a medium-sized guinea-pig. Then take 0.1 c.c. of the strong toxin and ascertain the amount of antitoxin necessary to neutralize it. Suppose it is 0.0025 c.c. Then this 0.0025 c.c. of the antitoxic serum is said to contain *one immunizing unit, or 1 c.c. of this antitoxin contains 250 immunizing units.* If in a case of diphtheria in a cat it is known from experience that it will take 500 immunizing units to cure it, it will be necessary to inject 2 c.c. of the antitoxic serum or use a serum four times as strong and give 0.5 c.c. to get the same effect. In order to protect a horse prophylactically against tetanus it is necessary to give 10 c.c. to 20 c.c. of a strong tetanus antitoxin, which dose contains several thousand immunizing units.

IMMUNITY.

Definition.—Immunity may be defined as the ability of a higher animal organism to resist invasion by, and multiplication of, pathogenic microörganisms and to neutralize their poisonous products. The agencies to which the protection is due have already been named and explained. They are the phagocytic cells, the opsonins, antitoxins, agglutinins, lysins, precipitins, and a number of others.

Congenital Immunity.—Certain animals may possess a congenital natural immunity. For instance, many warm-blooded mammals, such as cattle, sheep, mice, rabbits, guinea-pigs, are susceptible to anthrax infection, while adult dogs and rats possess quite a strong, though not absolute, natural immunity against this infection. The horse and man are susceptible to glanders; cattle are immune. Man is susceptible to typhoid bacillus and cholera spirillum infections, while all our domestic animals are immune, as far as natural infection is concerned.

Acquired Immunity.—Persons who have one attack of the following diseases are generally immune against a second attack, viz., measles,

scarlatina, typhoid fever, smallpox. The same is true of animals after once having had hoof-and-mouth disease, rinderpest, and distemper (in dogs). This is called a natural acquired immunity.

Immunity may also be acquired by *artificial means.* When an animal is inoculated with an attenuated virus or vaccine the aim is to produce a comparatively mild attack of the disease. This mild attack protects the animal for some time against another attack of the same infection, be it severe or otherwise. This procedure, known as (artificial) *active* immunity, has made the animal immune. Example, anthrax vaccination with the attenuated virus.

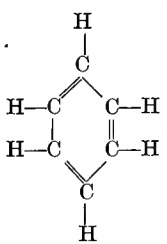
Passive Immunity.—Besides vaccination, immunity may also be conferred by injecting into an animal the antitoxic or immune serum of another animal. This type is called *passive immunity,* and is largely used in preventing tetanus or diphtheria by the subcutaneous injection of tetanus and diphtheria antitoxins.

Simultaneous Method.—In some cases an *active immunity* is produced by the so-called simultaneous method, *that is, by an injection of a virus or vaccine and an antitoxin at the same time.* An active immunity generally lasts much longer and protects much better than a passive immunity, but in some cases it is quite dangerous to inject even a very small but still effective dose of a virus. Therefore, in order to lessen the danger of severe sickness and death the antitoxin is injected at the same time. The simultaneous method is employed in the following cases: In inoculating horses for tetanus with both tetanus toxin and antitoxin; in immunizing cattle against rinderpest by injecting simultaneously blood from a virulent case and serum from an animal that has recovered from the disease; by immunizing cattle against anthrax by injecting at the same time an attenuated bacillus and the immune serum from a mule. This method is also used in immunizing swine against hog cholera.

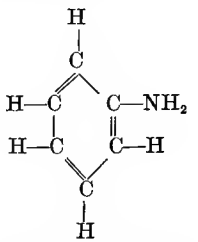
Ehrlich's Side-chain Theory of Immunity.—It has previously been stated that toxin and antitoxin apparently unite inside and outside of the body, neutralizing each other somewhat like ordinary chemicals, such as acids and alkalies. It can be shown that the experimental union of toxin and antitoxin always takes place in definite amounts, and that it occurs more quickly in concentrated solutions and at higher temperatures (35° to 40° C.). These facts led Ehrlich to suspect that the chemically highly complex toxins and antitoxins possess certain molecular groups having toward each other a high degree of chemical affinity, which causes them to unite somewhat as acids and alkalies do whenever there is a chance for a union. From this fundamental idea Ehrlich developed his celebrated side-chain theory of immunity. In order to understand this it is well first to explain what is meant by a side-chain in organic chemistry.

Benzene Ring.—According to the hypothesis of Kekule, the organic compounds of the aromatic group of which benzene or benzole is a

representative, have their carbon atoms arranged in a ring. Carbon is a tetravalent atom, that is, each atom has four chemical affinities which can be satisfied by other atoms in forming various chemical compounds. The formula of benzene, which is a stable chemical compound, in which all affinities are satisfied, is C_6H_6. Its chemical structure, according to Kekule's hypothesis, is explained by assuming that the tetravalent carbon atoms form a ring in which each of the carbon atoms is united with one neighbor by two affinities and with the other neighbor by one affinity. This leaves for each carbon atom one affinity free to which the hydrogen atom is united. The picture of the hypothetical benzene ring is shown in Formula A.



Formula A. Benzene.



Formula B. Anilin oil.

Each of the hydrogen atoms may be replaced by a more complicated molecular group, so that we may have, for instance, a body of the formula $C_6H_5NH_2$ (Formula B), or anilin oil, which is the basis of all anilin stains used in pathologic and bacteriologic work. In this body the group NH_2, which is attached to one of the carbon atoms, is called a *side-chain.*

According to the hypothesis of Ehrlich, the chemical substances of cells as well as of toxins and similar bodies contain side-chains, which by uniting in the animal body produce both poisonous effects and immunity. How this is accomplished according to the hypothesis will be shown. The chemical formulæ of the live cell substances, the toxins and the hypothetical side-chains, are not known. Therefore, in order to demonstrate graphically what is supposed to happen, figures which *represent the matter as a simple physical arrangement taking place between geometrical bodies* are employed.

Side-chains.—The side-chain theory assumes that a toxin has one side-chain which can attach itself to the side-chain of a cell. After this has occurred another side-chain of the toxin fastens itself to a second side-chain of the same cell. It is only after this second attachment has taken place that damage is done to the cell. In other words, the first side-chain only serves to anchor the toxin to the cell, and the second side-chain of the toxin produces the poisonous effect.

The four side-chains in this mutual process between the cell and the toxin have received special names.

Haptophore Group.—The side-chain which simply attaches the toxin to the cell.

Haptophile Group.—The side-chain of the cell to which the toxin becomes attached.

Toxophore Group.—The side-chain of the toxin which has the poisonous effect.

Toxophile Group.—The side chain of the cell to which the toxophore group of the toxin becomes attached, and by which attachment the damage is done.

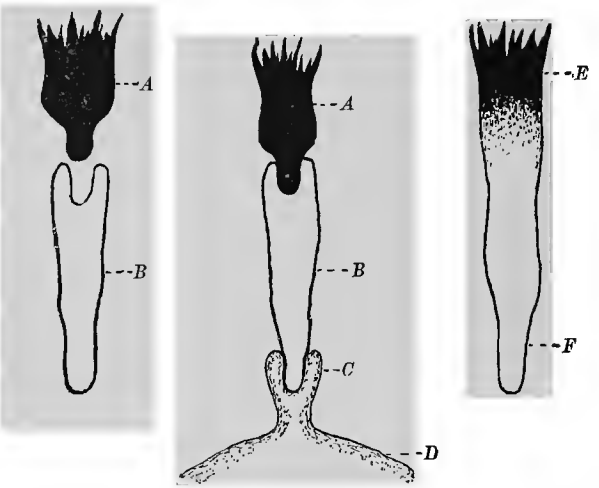
Receptors.—Side-chains of the cells which unite with the side-chains of the toxins are also called by a general term, the cell receptors. It must be assumed that the attachment of the side-chains of the toxin to the cell receptors destroys the latter, at least, so far as their character and existence as side-chains are concerned. It is a general observation in pathology that the destruction of physiologic parts is always followed by an attempt on the part of the organism to replace what has been lost. So if any liver or kidney cells have become necrotic the organism tries to replace them by new cells; this is called a *regeneration.* Very frequently an attempt at regeneration leads to the production of an *excess* of that which had been lost.

The side-chain theory assumes that whenever cell receptors have been made useless by toxins the cell not only replaces the lost receptors but that it produces them in great excess. So great does this excess become that the cell body cannot retain all the new receptors or new side-chains, and it must expel a large number of them into the blood serum and other juices of the body. If an animal which possesses a large number of such receptors in its blood serum becomes infected a second time by the same toxin, what occurs? Clearly, before the toxins have a chance to attach themselves to the cells they are caught, as it were, by the *free floating receptors* which prevent them reaching the cells and render them harmless. Thus the animal is protected against them. *A serum full of free receptors which will catch and unite with the toxins is called an immune serum or an antitoxic serum.* In fact, the free receptors are the *antitoxin* which unites with the *toxin* to protect the cells.

Toxoids.—It has been stated that the toxin possesses a *haptophore* and a *toxophore group.* This can be shown by the observation that solutions of toxins after some time lose much or all of their poisonous properties, and yet these poisonless non-toxic toxins may still be able to anchor themselves to cells and cause the formation of free receptors. In other words, they can still be used to manufacture an antitoxic immune serum in the body of an animal. The toxin has lost its toxophore side-chain or group, but it still possesses its haptophore side-chain or group. Such a toxin is called a toxoid. Ehrlich has introduced into the nomenclature of serum investigation the two symbols L_o and L_+. The letter L stands for the term *lethal* (deadly, fatal). The former symbol L_o designates a toxin-antitoxin mixture which is *completely neutralized,* and therefore will not kill

an animal. While the latter symbol L_+ designates a toxin-antitoxin mixture which contains *one fatal dose in excess* which will kill the

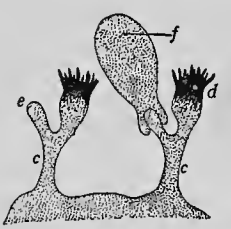
FIG. 33



Graphic representation of receptors of the first and third orders and of complement as conceived by Ehrlich: *A*, complement; *B*, intermediary or immune body; *C*, cell receptor; *D*, part of cell; *E*, toxophorous group of toxin; *F*, haptophorous group. (Park.)

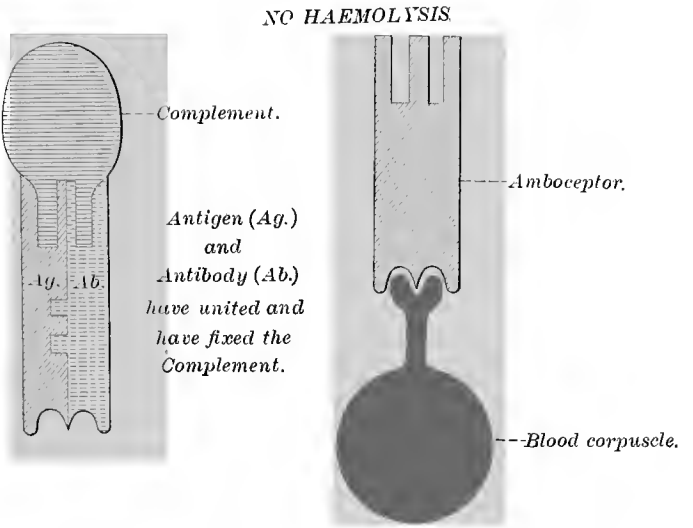
experimental animal within a few days. If a toxin is freshly prepared and its minimum fatal dose ascertained it will be found that a certain amount of antitoxin is required to neutralize it completely. If 100 fatal doses are taken it will require 100 protective doses of the antitoxin to produce a completely neutralized mixture (L_o). However, when the toxin gets older it can be shown that instead of 100 protective doses only about 80 are required to prevent a fatal effect. In the case of the fresh toxin L_+ minus L_o is equal to *one* fatal dose; but after the toxin has gotten older L_+ minus L_o is equal to apparently 21 fatal doses. The reason for this peculiar behavior has been already explained above. The toxin that is part of it has lost its *toxophore group*, but has retained its *haptophore group*, and with it its combining power toward free cell receptors on antitoxins. The toxin has been changed into a toxoid.

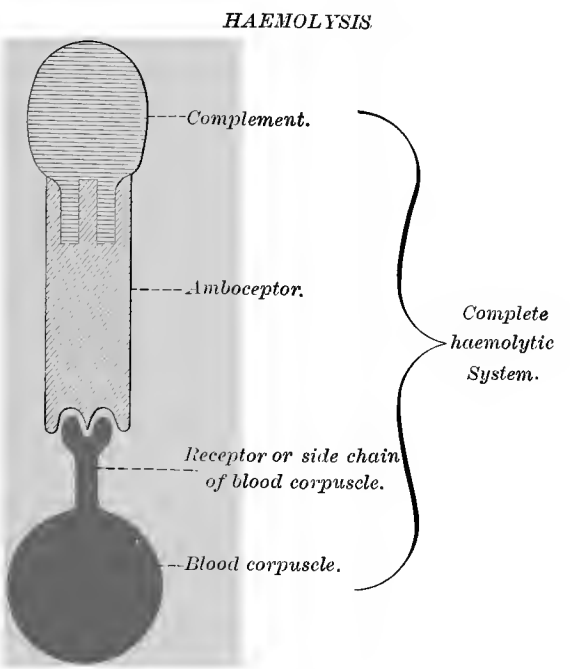
FIG. 34



Receptors of the second order are pictured in *c*. Here *e* represents the haptophore group, and *d* the zymophore group of the receptor, *f* being the food molecule with which this receptor combines. Such receptors are possessed by agglutinins and precipitins. It is to be noted that the zymophore group is an integral part of the receptor. (Park.)

PLATE II


NO HAEMOLYSIS
Complement.
Ag.
Ab.
Antigen (Ag.) and Antibody (Ab.) have united and have fixed the Complement.
Amboceptor.
Blood corpuscle.


HAEMOLYSIS
Complement.
Amboceptor.
Receptor or side chain of blood corpuscle.
Blood corpuscle.
Complete haemolytic System.

The conditions of interaction and neutralization of antigen and antibody are not always as simple as in the case of toxins and antitoxins. They are more complicated with reference to other antibodies, and it will be well to look into one important example of this kind.

Hemolysins.—If we inject into the body of a guinea-pig at proper intervals and in proper doses cholera spirilla the animal develops an immune serum which has the property of dissolving cholera spirilla. This property of the immune serum is due, as stated before, to a particular kind of antibody known as *lysins.* If we inject into a rabbit the red blood corpuscles of a sheep the former will develop an immune serum which has the property of dissolving sheep's corpuscles. This property of the injected or *sensitized rabbit's serum* is due to an antibody of the lysin type called a *hemolysin.* It consists, as can easily be shown, of two bodies which can be separated and studied separately. One of these two constituents which make up the complete hemolysin is already found in the normal serum of most animals; the other constituent antibody is only found in the immune serum (the serum of the sensitized rabbit) or at least found there only in large amount. The body found in normal serum is called the *complement,* and the other the *immune body* or *amboceptor* (why this latter name is used will be explained presently). The complement is an antibody which is very easily destroyed and cannot stand a temperature of 56° C. if applied to a serum containing it for thirty minutes. Hence, *the complement is said to be thermolabile.* The amboceptor, on the other hand, can well stand heating for thirty minutes at 56° C.; therefore, it is said to be *thermostabile.* If an immune serum containing a hemolysin is heated for thirty minutes at 56° C., destroying the complement, but not the amboceptor, it is said to be *inactivated,* because it now cannot bring about hemolysis. If, however, some normal non-heated serum which, as stated, contains the complement is added to an inactivated serum the immune serum is again reactivated because it can once more bring about hemolysis (solution of the red blood corpuscles). This method of inactivating the immune serum and using it in connection with reactivating normal serum makes it possible to study the amboceptor and the complement separately and to learn how they act, how they anchor themselves to the red blood corpuscles, and how, by so doing, they bring about hemolysis. The process by which this occurs is the following: The amboceptor has *one group,* or side-chain, which fastens itself to a receptor of the red blood corpuscle, and a *second group,* or side-chain, by which it attracts and anchors to itself the complement. Only after the amboceptor has become united to both the complement and the red blood corpuscle can the solution of the latter (hemolysis) take place. The union of (1) red blood corpuscle, (2) amboceptor, and (3) complement is called *a complete hemolytic system or chain.* This explains why the immune body in

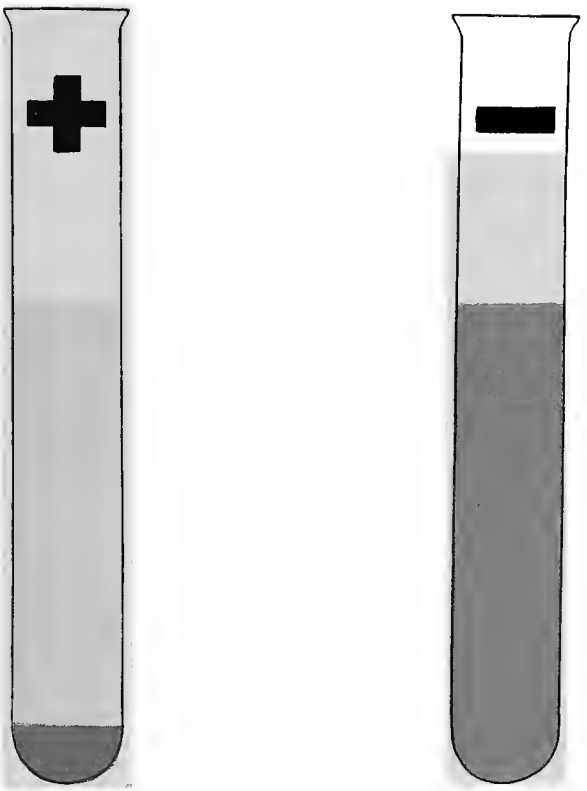
hemolysins has been called *amboceptor*, which means, literally translated, "*double catcher*," or "*double taker.*"

Deviation of Complement.—To a solution containing a hemolytic amboceptor and a complement, something may be added which will unite with the complement, and the solution will then loose its property of dissolving blood corpuscles (to produce hemolysis). The complement has united to something else, and can no longer unite with the amboceptor, a complete hemolytic system cannot be formed, and, therefore, hemolysis cannot take place. *In other words, complement deviation prevents hemolysis.*

Complement deviation can be brought about by several means; for instance, by the use of anticomplements or by the presence of *an antigen uniting with its antibody.* These two, when united, generally form a combination which will anchor to itself the complement and so prevent hemolysis. Complement deviation has been here explained somewhat at length, because it has become a most important method in practical serum diagnosis. The principle was discovered by Bordet and Gengou. They found that if an antigen be permitted to unite with its specific immune body or immune amboceptor, something is formed by the union which will attract to itself the complement of a hemolysin, so that the latter can no longer bring about a solution of the red blood corpuscles. This peculiar occurrence or phenomenon may be used to detect antibodies. Suppose the presence of certain antibodies is suspected in the blood serum of a person or animal they can be detected in the following manner: Add to the blood serum the antigen of the suspected antibodies and a hemolytic complement. Later, add the hemolytic amboceptor and some red blood corpuscles. If solution of the latter occurs the complement is not deviated, because the suspected antibody is not present, and could not unite with the antigen, and could not, therefore, produce complement deviation. If, however, there is no hemolysis of the red blood corpuscles it clearly shows that the suspected antibody is present. It has united with its antigen and they in their union have attracted the complement, which is no longer present in the free state; hence, the hemolytic chain, composed of red blood corpuscles, hemolytic amboceptor, and hemolytic complement, *cannot be formed and hemolysis cannot occur.*

The Wassermann Test.—Syphilis in man is often very difficult to diagnosticate, and it is still more difficult to determine whether the disease has been cured or whether it is still going on in a latent form. The phenomenon of complement deviation under properly arranged tests has, however, furnished a means of an almost absolutely infallible diagnosis. *Since the same principle may be applied to diseases of domestic animals, and, perhaps, particularly to dourine or horse syphilis, also very difficult to diagnosticate,* it is important to know in detail the steps of the serum diagnosis of syphilis, which has assumed such great importance in human medical practice. The author has

PLATE III



Positive. Negative.

Wassermann Test.

for some time tried to find a chance to apply his test to dourine of horses, but, unfortunately, no occasion has offered. Very probably the test could be made in an identical manner, but this would, of course, have to be tried out first experimentally. The serum test for the diagnosis of syphilis was devised by Wassermann and his co-workers, Neisser and Bruck. It is generally known simply as the Wassermann test.

The following reagents and preliminary steps in their proper arrangement, dilution, etc., are necessary:

1. The red blood corpuscles of the sheep are used for the final hemolytic tests. They must be washed free from all blood serum. The blood is obtained either from the jugular vein of the live animal or from a slaughter house at the time when the animal is killed. It is best to have the blood run into a sterile vessel, though this is by no means absolutely necessary. It is at once defibrinated by beating it with a bundle of wires or glass rods or by shaking it with some fragments of glass or glass pearls. After the fibrin has coagulated there remains an intensely red fluid containing the serum and the corpuscles. Some of this fluid is mixed in the proportion of one to ten with physiologic salt solution. Two centrifuge tubes are filled with the mixture, and these are centrifuged for from five to ten minutes. There is now formed a deposit of corpuscles and an upper layer of clear fluid. The latter is pipetted off; the tubes are again filled with normal salt solution, shaken so that the corpuscles and the fluid are mixed; they are centrifuged again and the clear fluid is pipetted off once more. This is done at least three times. Finally, about one part of the washed sheep's corpuscles is mixed with nineteen parts of physiologic salt solution and shaken until an emulsion is obtained. *The latter is called the 5 per cent. emulsion of washed sheep's corpuscles.*

2. In order to make the test properly it is necessary to have a *strong hemolytic amboceptor.* This is obtained by "*sensitizing a rabbit against sheep's corpuscles*" in the following manner: The abdominal region of the rabbit is shaved and cleansed antiseptically. The animal is then held up by two assistants in such manner that its head hangs down and the hind legs, which are spread out, point upward. In this position the rabbit's intestines fall toward the diaphragm and there is little danger of injuring them in the next step, which consists in injecting into its peritoneal cavity about 10 c.c. of a 5 per cent. physiologic salt emulsion of washed sheep's corpuscles (this must have been freshly prepared under aseptic precautions and the injection must be made with a sterile syringe). This procedure is repeated three or four times or oftener at intervals of about ten days. The blood serum of a rabbit so treated will have, as a rule, a very strong hemolytic power toward sheep's corpuscles. Since the serum is sometimes deficient in spite of the injection treatment, it is well to draw some blood from an ear vein and test it

before all of the rabbit's serum is collected. If the preliminary trial shows a strong hemolytic power, the rabbit is killed by cutting both carotid arteries and allowing the blood to flow into a sterile cylindrical vessel. The blood is allowed to coagulate, and is then placed on ice for a number of hours, after which the serum is removed from the coagulum. If the serum is not perfectly clear and still contains some red blood corpuscles, it must be centrifuged. The clear serum is finally pipetted off. This is next heated on a water bath at 56° C. for thirty minutes. By so *inactivating* the serum, we destroy, as explained before, the hemolytic complement, so that the rabbit's serum will now *alone not hemolyze sheep's corpuscles.*

3. The complement necessary to produce hemolysis is obtained in the blood serum of the guinea-pig. An animal of this kind is bled to death by severing the carotid arteries. The blood is collected at once in some centrifuge tubes, and after coagulation is centrifuged and the serum pipetted off from the clot. The complement in the guinea-pig's serum is very easily destroyed, by simply allowing the serum to stand at room temperature. If the serum cannot be used at once it must be placed in the refrigerator, and if it is to be kept for more than twenty-four hours, it must be frozen hard in a mixture of salt and ice and kept in a thermo bottle in a freezing mixture.

4. The rabbit's inactivated serum is now tested in the following manner:

(*a*) Take a number of test tubes and place into each ten drops of a 5 per cent. physiologic salt solution emulsion of washed sheep's corpuscles.

(*b*) Add to them one drop of the guinea-pig's serum and nine drops of salt solution.

(*c*) Now add to the different test-tubes varying amounts of the inactivated rabbit's blood serum plus enough salt solution to make ten drops, say as follows:

To tube No. 1, add one drop.
To tube No. 2, add one-half drop.
To tube No. 3, add one-fourth drop.
To tube No. 4, add one eighth drop.
To tube No. 5, add one-twelfth drop.
To tube No. 6, add one-sixteenth drop.

These additions have to be made exactly by previously diluting some of the inactivated rabbit's serum accurately; for instance, dilution No. 6 would be made by adding to one drop of serum fifteen drops of salt solution and taking of this dilution one drop plus nine drops of salt solution to test-tube No. 6.

(*d*) Finally, add to each tube twenty more drops of the salt solution, so that each tube contains exactly fifty drops. Shake them well and place them all for thirty minutes into the incubator. Examine after they have been incubated one-half hour.

Suppose that at this time all the tubes except No. 6 show complete hemolysis. This one shows only partial hemolysis. This means that one-twelfth of a drop of the inactivated blood serum of the rabbit will be sufficient for complete hemolysis with the quantities used in the test. It is customary to use in the final determining tests about three times the minimum amount of rabbit's serum which will bring about complete hemolysis. Hence, if the final determining tests are arranged in the same proportion as the tests made to ascertain the titre (or strength) of the rabbit's serum, one-quarter of a drop of this serum would be used.

5. It is now necessary to make another test by adding to ten drops of a 5 per cent. washed sheep's corpuscles emulsion, diluted with forty more drops of salt solution, one drop of rabbit's serum and placing this tube into the incubator for several hours. At the end of this time there must not be any hemolysis at all. This will show that the rabbit's serum has been inactivated properly, and that it contains no more complement, the latter has been entirely destroyed and cannot interfere in the final test. The inactivated rabbit's serum must be kept in the refrigerater, where it will keep for several months.

6. Another necessary reagent is an extract which will contain the antigen of the suspected *syphilitic antibodies* (or syphilitic amboceptor) *the presence or absence of which in the blood serum to be examined* is to be determined by the test. This antigen cannot be prepared from a pure culture of the organism causing syphilis, because so far it has been impossible to obtain it in pure culture. It can be prepared, however, from the liver of a newborn child dead from congenital syphilis. This liver is cut up into small pieces and one part by weight of liver is rubbed up very thoroughly with five parts of absolute alcohol. The mixture is then shaken in a shake machine for twelve to twenty-four hours, allowed to stand, then filtered and the clear filtrate known as the alcoholic antigen extract is ready for use.

7. Complement deviation in the case of syphilis is *not an absolutely strictly specific reaction*, which means that the syphilitic antigen extract can be replaced by some other preparations which will do the same work, and unite with the syphilitic antibodies and deviate the complement. The substitute most commonly used in place of the luetic antigen is *an alcoholic extract of guinea-pig's heart*, which is prepared as follows: After the animal is dead the heart is removed, washed in physiologic salt solution, dried between filter papers, and weighed. The heart is then cut up, and the parts dropped into a mortar which contains washed sterile quartz sand; add fifty times the weight of the heart of 95 per cent. alcohol and triturate with the alcohol and sand until the heart has been very finely divided. The entire mixture is next removed from the mortar to a flask or other suitable glass vessel and heated on a water bath for three to four hours at 60° C. After cooling, the evaporated alcohol must be made up to the original

bulk. It is now filtered off from the sand, etc., and the clear alcoholic extract, placed in a tightly glass-stoppered bottle, is ready for use at any time. This alcoholic extract of guinea-pig's heart keeps for many months at room temperature, provided it is protected against evaporation and contamination.

The blood sera used for the tests are the serum from the patient to be examined, the serum from a healthy person and a serum from one who is in the early stages of syphilis. The two latter sera are, of course, used as controls in connection with tests of the patient's serum. Blood is obtained from the three persons with a sterile, all-glass, large hypodermic syringe, the barrel of which will hold from five to ten cubic centimeters of blood. After the latter has been drawn under all aseptic precautions from a vein of the arm, it is at once placed in a centrifuge tube. After coagulation the clot is loosened from the walls of the tube with a platinum wire, the tube is centrifuged, and the serum pipetted off. If not clear, it is centrifuged a second time. After the three clear sera have been obtained they are heated for thirty minutes at 56° C. on a water bath. This *inactivating* is done to *destroy the complement* present, but does not interfere with the syphilitic antibody or syphilitic amboceptor.

8. Everything is now ready to make the test, in the following dilutions. The alcoholic antigen extract is used in a dilution of two in ten, with physiologic salt solution. The rabbit's inactivated serum which contains the hemolytic amboceptor is used in a dilution of one drop in forty or one-quarter of a drop in ten of the salt solution. The reagents are now employed as follows:

(*a*) Alcoholic extract of antigen (dilution two in ten).

(*b*) Three inactivated sera, one from the patient, two controls.

(*c*) The fresh unheated serum from the guinea-pig containing the hemolytic complement.

(*d*) The inactivated rabbit's serum (dilution one in forty) containing the hemolytic amboceptor.

(*e*) The 5 per cent. washed sheep's corpuscles salt solution suspension, containing the sheep's corpuscles, on which hemolysis will be tried.

The test is now made as follows:

Prepare six test-tubes and label as follows: Patient No. 1; Patient No. 2; Positive control, No. 1; Positive control, No. 2; Negative control, No. 1; Negative control, No. 2. Also label the tubes in this order from No. 1 to No. 6, consecutively. Now add to each tube as follows:

To No. 1 (patient No. 1), 10 drops alcoholic antigen extract, plus 2 drops of patient's serum, plus 8 drops of salt solution, plus 1 drop of guinea-pig's serum, plus 9 drops of salt solution.

To No. 2 (patient No. 2), 10 drops of salt solution, plus 2 drops of patient's serum, plus 8 drops of salt solution, plus 1 drop of guinea-pig's serum, plus 9 drops of salt solution.

To No. 3 (positive control No. 1), 10 drops of alcoholic antigen extract, plus 2 drops of serum of syphilitic, plus 8 drops of salt solution, plus 1 drop of guinea-pig's serum, plus 9 drops of salt solution.

To No. 4 (positive control No. 2), 10 drops of salt solution, plus 2 drops of syphilitic serum, plus 8 drops of salt solution, plus 1 drop of guinea-pig's serum, plus 9 drops of salt solution.

To No. 5 (negative control No. 1), 10 drops alcoholic antigen extract, plus 2 drops of healthy person's serum, plus 8 drops of salt solution, plus 1 drop of guinea-pig's serum, plus 9 drops of salt solution.

To No. 6 (negative control No. 2), 10 drops of salt solution, plus 2 drops of healthy persons serum, plus 8 drops of salt solution, plus 1 drop of guinea-pig's serum, plus 9 drops of salt solution.

We have now three sets of tubes, each set containing the serum of a different person, and in each set one tube with alcoholic antigen extract and one set without this extract, its place being taken by physiologic salt solution. There must be another set of controls prepared from the sera to find out whether the extract is of the proper strength. In these controls the alcoholic extract is present in the following proportions: 1 to 10, 2 to 10, 4 to 10, 6 to 10. The extract should be of such strength that 1 to 10 is strong enough to inhibit hemolysis with a serum from a case of syphilis; 4 to 10 should be of such a strength that the presence of a syphilitic antibody is necessary to prevent hæmolysis, and 6 to 10 should be strong enough to prevent it alone. These additional tests need not be made every time, but just often enough, so that the alcoholic antigen extract is always kept under control. Another point about this extract is the following: Since it contains in the proper dilution about 25 per cent. alcohol, its drops are smaller than the drops of the purely watery fluids used, hence it is necessary to use a special dropper for the alcoholic extract which has been tested out, or if the same dropper or pipette is used, it is necessary to take from twelve to thirteen drops of the dilute alcohoic extract for each ten drops of the watery solutions. All reagents must be used so that they represent in the salt solutions equal amounts, namely, ten drops.

After the test-tubes have been prepared they are placed in the incubator for thirty minutes. If there is present in any test-tube a combination of antigen and syphilitic antibody, these will, *during the half-hour in the incubator, unite and attract and fix to themselves the complement* which is present in each tube.

After thirty minutes the six test-tubes and the other controls which may have been made are removed from the incubator and to each one is added:

10 drops of the diluted inactivated rabbit's blood serum containing the hemolytic amboceptor.

10 drops of the 5 per cent. washed sheep's corpuscles salt solution suspension.

The tubes are well shaken and replaced in the incubator for one to one and one-half hours. They are then taken out and the result may be recognized at once, or, better, the tubes are placed overnight in the refrigerator. This will enable all undissolved corpuscles to sink to the bottom of the tubes and a very characteristic unmistakable picture is formed. Hemolyzed tubes show a uniformly transparent red stained fluid. Non-hemolyzed tubes show a sediment of red blood corpuscles at the bottom and a perfectly clear supernatant salt solution on top. The result of the test in case the suspected patient has syphilis will be:

Tube No. 1 (patient No. 1), no hemolysis.
Tube No. 2 (patient No. 2), hemolysis.
Tube No. 3 (positive control No. 1), no hemolysis.
Tube No. 4 (positive control No. 2), hemolysis.
Tube No. 5 (negative control No. 1), hemolysis.
Tube No. 6 (negative control No. 2), hemolysis.

The result is explained as follows: The patient has syphilis, hence in tube No. 1 the antigen (alcoholic extract) and the syphilitic antibodies united; they deviated the complement and hemolysis could not take place. The same conditions prevail as to tube No. 3; the blood came from a person known to have syphilis. No. 5 contained the blood of a healthy person, hence the antigen (alcoholic extract) could not unite with syphilitic antibodies, since none were present; consequently, hemolysis took place. In tubes Nos. 2, 4, and 6 no alcoholic antigen extract was added; hence, even if syphilitic antibodes are present, as in tubes Nos. 2 and 4, they had no antigen to unite with, and hence could not deviate the complement, and hemolysis took place.

If the patient does not have syphilis, hemolysis will, of course, take place in Tube No. 1; since there are no syphilitic antibodies present they cannot unite with the antigen, and the complement will not be deviated.

Anaphylaxis and Hypersusceptibility.—A very peculiar occurrence, not as yet fully understood, has been observed and studied experimentally by Arthus, Theobald Smith, Rosenau and Anderson, and others. If an animal receives a very small hypodermic or intraperitoneal injection of an alien or heterologous blood serum, that is, a blood serum from an animal of a different species, and after the lapse of about three weeks a larger dose (for instance, several cubic centimeters) of the same blood serum, a very grave condition frequently develops. An animal so treated may show difficulty in respiration, rapid pulse, convulsions, and death. There has evidently been established in the animal in consequence of the first small dose of the alien serum a hypersusceptibility to this serum. However, if animals showing this complex of symptoms do not die they rapidly get over the attack and are soon well again. Anaphylaxis can be produced both actively by injecting into a guinea-pig an alien serum

(for example that of the horse) and passively by taking the serum of the same guinea-pig, after two or three weeks, and injecting it into another guinea-pig, producing in it the same anaphylaxis or hypersusceptibility against the alien serum.

Sudden Unexplained Loss in Antitoxic Value.—Another occasional peculiar occurrence difficult to explain with our present knowledge of the details of the processes of immunity is the following:

A horse may have been highly immunized (hyperimmunized) against tetanus toxin. The blood serum of this horse shows an enormous amount of antitoxin—in other words, it shows in each cubic centimeter a high figure of immunity units. The animal receives another large dose of tetanus toxins. This ordinarily would cause the antitoxic value of its serum to rise still higher, or, at least, if the limit has been reached to remain stationary. Instead of this the antitoxic value sinks enormously, the toxins are not properly neutralized, and the horse gets very sick and may even die. There is no satisfactory explanation for this peculiar occurrence which apparently opposes all dicta of the theories of immunity.

QUESTIONS.

1. What is an antitoxin? What effect has it upon a toxin?
2. How can this effect be demonstrated?
3. What happens if we inject cholera spirilla into the peritoneal cavity of an animal not very susceptible to them?
4. What is an agglutinin? What is a lysin?
5. What properties does a rabbit's blood serum acquire if we inject into this animal human blood serum several times at intervals?
6. What is a precipitin? Describe the procedure necessary to produce precipitins for horse's blood serum in the body of a rabbit.
7. What practical use has been made of the precipitin test in forensic medicine?
8. Give a definition of the term antibodies.
9. What is an antigen?
10. What are the antibodies against tetanus and diphtheria toxins? How prepared and obtained?
11. What is the paraenteral method of producing antibodies?
12. What is meant by virulency; what by attenuation? How can virulent bacteria or their toxins be attenuated?
13. What is meant by a vaccine?
14. What is an immune serum?
15. What is meant by the term immunity?
16. What is congenital natural immunity? What is naturally acquired immunity?
17. What is artificial immunity? What is the difference between active and passive immunity?
18. What is the simultaneous method of immunizing an animal? Name some diseases against which this method is used.
19. What circumstances influence the union between toxin and antitoxin?
20. What is the benzole ring of Kekule?
21. What does the term side-chain mean as used in organic chemistry?
22. Explain the terms: haptophore, haptophile, toxophore, toxophile, side-chains.
23. Explain the interaction of these side-chains toward each other.
24. What generally happens if tissue elements are destroyed?
25. What is a cell receptor?
26. What happens according to the side-chain theory if cell receptors are destroyed?

27. What are the names given to the free side-chains contained in the blood serum? What is their relation to toxins liberated in the body of an infected animal?
28. What is a toxoid? Under what conditions is it formed?
29. What is a hemolysin? Is it a simple or a compound antibody? What enters into its formation?
30. What is the meaning of thermolabile and thermostabile? What does inactivating mean?
31. What is an amboceptor? Why so named?
32. How can a previously inactivated serum be reactivated?
33. What is a complete hemolytic system or chain?
34. How can the hemolytic complement be deviated or fixed?
35. How can a rabbit be sensitized so that its blood serum will bring about hemolysis of sheep's corpuscles?
36. Describe the method of washing sheep's corpuscles.
37. How is the hemolytic complement for tests in hemolysis generally obtained?
38. How must this complement, which is easily destroyed, be preserved?
39. Describe the method of inactivating sensitized rabbit's serum.
40. How are the clear sera from animals or man obtained? How are they treated before use?
41. In what proportions and dilutions are the reagents in a test for complement deviation used?
42. How is the antigen extract in Wassermann's serum test for syphilis prepared?
43. Describe in detail the steps of the Wassermann test.

CHAPTER VIII.

METHODS OF OBSERVING BACTERIA—THE USE OF THE MICROSCOPE AND ACCESSORIES.

Cultures.—It is absolutely necessary in the study of pathogenic bacteria to obtain each definite species free from any other live organisms. When such a preparation is successfully made whether of a pathogenic or other bacterium it is called a pure culture. It is generally composed of small discrete masses called colonies, and these in turn are formed by innumerable individual bacilli which in their entity make up the pure culture. When bacteria have grown abundantly on a properly prepared artificial culture soil they often appear as one continuous mass of the growth and the individual colonies are no longer distinguishable. They were present very early in the course of the development, but have become confluent. If the bacterium has originally been inoculated into the culture soil in a very dilute form individual colonies can always be seen.

Microscopic Study.—Several of the properties and characteristics of pathogenic bacteria in pure cultures can be studied with the naked eye, such as the varying degrees of moisture or dryness, the smooth or granular surface of the growth, its color, its power to liquefy certain culture media, etc. In order to observe the individual bacterium, however, a microscope is required with one low and one high power lens, and an especially powerful illuminating apparatus. The preparation of pure cultures is much more difficult and time-consuming than the microscopic study of bacteria, after they are once obtained in a pure state; hence, the student should first familiarize himself with the use of the microscope in observing bacteria in the stained and unstained condition. This study will, therefore, be considered prior to the subject of culture media, pure cultures, and the sterilization methods necessary to obtain them.

Source of Light.—In the microscopic study of bacteria, natural, reflected, diffuse light is employed during the day, best obtained at a window with a northern exposure. When artificial light is used, an ordinary coal-oil lamp or gas burner will serve the purpose, an Argand burner is better, and a Welsbach light is best of all. An Edison incandescent lamp is not a good light, and if used at all should have a bulb of frosted glass, to prevent the incandescent film from disturbing the field of vision.

The Microscope.—The modern microscope consists of a stand forming the foot or base and carrying the stage and a brass tube

containing the optical apparatus, which can be moved up and down on a supporting arm from an upright pillar of the stand. The raising and lowering of this tube is accomplished by a rack and pinion, worked by a large screw-head on either side. This arrangement is called the coarse adjustment. In the outer tube is an inner tube which can be drawn out by hand. This inner tube is graduated in millimeters, and if the lower end of the outer tube is provided with a so-called revolver or nosepiece the inner tube should be drawn out to the mark 16 cm. or 160 mm. The microscope will then be so adjusted that the distance from the upper lens of the eyepiece to the objective is 170 mm. (170 millimeters = 17 centimeters). This is the distance to which the higher power objectives are corrected and at which they work best, giving the clearest picture of the object under observation.

There is, in addition to the coarse adjustment, the so-called *fine adjustment* worked by a micrometer screw, placed either on top or at the sides of the centre pillar of the instrument. This raises and lowers the draw tube only very slightly so that it can be adjusted to the one-hundredth part of a millimeter.

Condenser.—One of the most important parts of the instrument is the substage or Abbe condenser,[1] or illuminating apparatus. This is attached below the central opening of the microscope stage in such a manner that it has considerable movement in a vertial plane, allowing the upper lens of the condenser to be on a level with the stage when desired. The importance of this vertical movement will be seen presently. The substage condenser consists of three parts, namely, a reflector, an iris diaphragm and the condenser lens proper.

The reflector is a circular disk suspended so that it can revolve around the median axis of the equatorial plane, and has two surfaces, a plane mirror and a concave mirror. The condenser proper, which forms the upper part of the illuminating apparatus, consists of a system of lenses. Between the reflector and the condenser lens is an iris diaphragm. The word diaphragm means a partition wall, and in the modern microscope this wall between the reflector and the condenser opens and closes in a concentric manner like the iris of the eye, hence the name. Those who have done photographic work with the camera understand the workings of the iris diaphragm; it can be adjusted so that it forms a large opening, admitting a powerful bundle or pencil of rays of light, or it can be closed through all the intermediate stages to a pinhole opening admitting very little light. Some of the modern microscopes possess a second iris diaphragm placed above the substage condenser lens. This second diaphragm is used in place of the lower one when the condenser lens has been removed. When instruments are equipped with Abbe condensers the plane mirror of the reflector should be used and not the concave mirror.

[1] Called Abbe condenser because invented and perfected by Professor Abbe, of Jena.

Objectives and Eyepieces.—The most important part of the modern compound microscope is the objective and next to it the eyepiece. Upon these, particularly the former, depends the clearness of definition and details of the image obtained.

Refraction.—The natural law upon which the whole construction of the optical parts of the microscope (objectives and eyepieces) depends is that rays of light are deviated from their course when they travel from a transparent medium of a certain density into a transparent medium of a different density. This deviation from their path, which occurs according to very definite mathematical rules, is called refraction. Objectives and eyepieces can be so arranged that the light after being refracted forms an enlarged or magnified image in the eye of the observer. Objectives are of low, of medium, and of high magnification.

Aberration.—High-power objectives or lenses must be corrected to overcome two sources of defects which interfere with the clearness of the image. White light is composed of a number of component colors (the colors of the spectrum or rainbow) and the rays of different colors are refracted in a different manner by refractive media. Hence, they furnish an indistinct picture, not true in color, and which is confused by the appearance of color rings. This defect of high-power lenses is called their chromatic aberration. The other defect is due to the very strong curvature and very small radius. A lens so constructed will refract the rays at its periphery (margin) differently from the other rays, forming indistinct pictures. This defect is called its spherical aberration. Both the spherical and the chromatic aberration can be corrected by a combination of lenses made up of glasses with a difference in their refractive index. But the more complete the correction the more difficult and delicate is the construction of these lenses, and, therefore, the greater the cost.

Focus.—Every microscopic objective is a system of lenses which acts as a convex lens, and parallel rays of light passing through it are refracted so that they meet in a single point called its main or principal focus. The distance of this point or focus from the central point of the lens is called its focal distance. In microscopic objectives the higher the magnification the shorter will be the focal distance, and the lower the magnification the longer this distance.

Image.—If an illuminated object is placed somewhere between the single and the double focal distance of a convex lens there is formed on the other side of it an enlarged or magnified real image of the object. In the use of the compound microscope the object to be looked at is placed within the proper focal distance from the objective and a real magnified image is then formed in the interior of the draw-tube. The eye sees this through the ocular or eyepiece, which again enlarges the real image in the draw-tube, and forms in the eye a highly magnified visual image. This is the optical principle of the construction and use of the microscope.

Lenses.—The objectives commonly used in work in histology, pathology, and bacteriology generally have focal distances of two-thirds inch, one-sixth inch, and one-twelfth inch (16 mm., 4 mm., 2 mm.), and they magnify, if used with the proper eyepieces, about 80, 400, and 800 times or linear diameters.

Dry Lenses.—The first two of these three lenses are used without the interposition of any fluid between the cover-glass of the preparation and the front lens of the objective, and are called dry lenses.

Immersion Lenses.—The third one, that which has the highest magnification and the shortest focal distance, is used with a drop of thickened cedar oil, called homogeneous immersion oil, interposed between the cover-glass of the preparation and the front lens of the objective. Lenses used in this manner are called homogeneous immersion lenses. High-power lenses are best constructed as immersion lenses for optical reasons. When a preparation is examined the light is first reflected upward by the mirror of the substage illuminating apparatus, it then passes the opening in the iris diaphragm, enters the condenser proper, and is focussed by it upon the microscopic object to be examined. From the latter the rays of light enter the objective of the microscope. In doing so, when a dry lens is used, the rays of light on leaving the cover-glass of the microscopic preparation, pass through a small amount of air before reaching the lower lens of the objective, and are refracted in such a manner that a considerable portion of the light is lost. This loss of light and the decrease in the angle of aperture under which the rays enter the objective cause loss of clearness and detail in the picture. If, instead of air between the cover-glass and the front lens of the objective there is a drop of thickened cedar oil, which acts toward light in the same manner as the cover-glass of the microscopic preparation, or, in other words, possesses the same refractive index as ordinary glass, there will be no loss of light, but a larger angle of aperture of the entering rays and a much better image. Therefore, high-power lenses are constructed as homogeneous oil-immersion lenses, and such a lens with a numerical aperture angle of 1.4 is better than one with a numerical aperture of 1.3. Homogeneous immersion lenses have a very short focal distance (2 mm. = $\frac{1}{12}$ inch); hence, if a thick cover-glass be used it will be impossible to bring the lens within the proper focal distance of the objects (bacteria) to be looked at. It is, therefore, necessary to use, in work with bacteria, the thinnest cover-glasses, that is, No. 1. No. 2 may sometimes answer, but it is not safe to use them, and the student should always see that he uses for work with bacteria the thinnest kind.

Focussing.—In order to see clearly with the microscope, it is always necessary to lower or raise the tube in such a manner that the object has the proper focal distance from the front lens of the objective. The proper adjustment must be judged by the eye. This manipulation of the instrument to obtain a clear picture is called focussing,

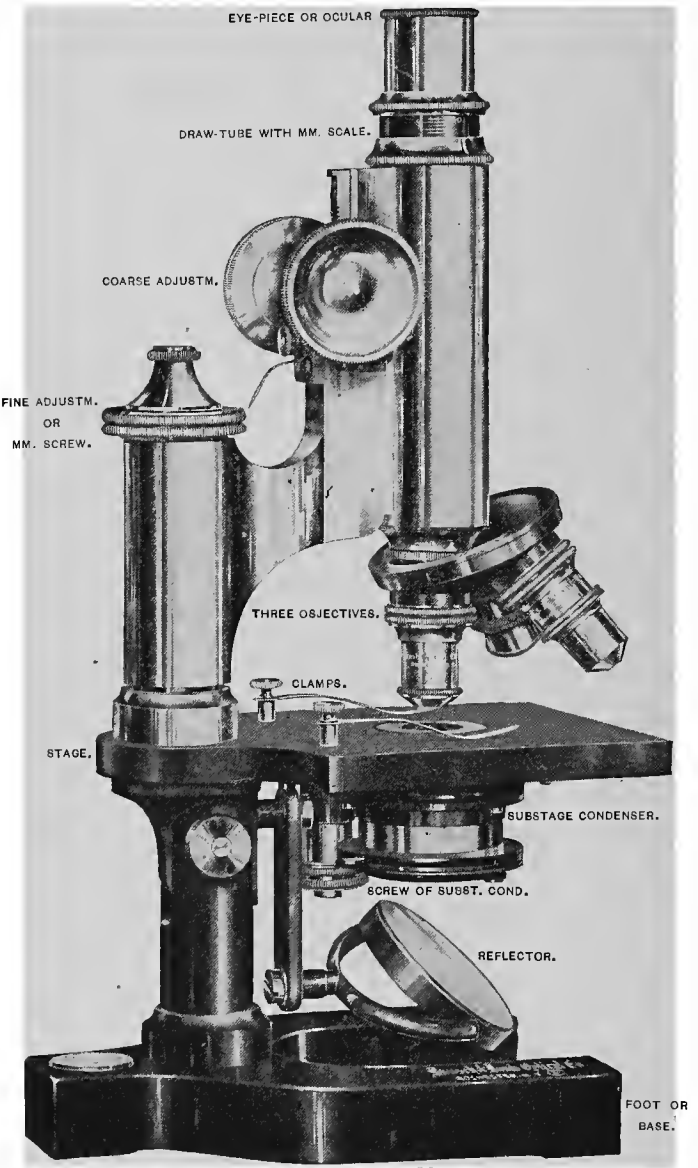
that is, getting the preparation or object into focus. The three objectives of a good modern microscope which are attached to the triple nosepiece or revolver are parfocal and correctly and identically centred. The term parfocal may be best explained as follows: Suppose an object is first focussed with the $\frac{2}{3}$-inch (16 mm.) or low-power lens. The nosepiece is now swung around so that the $\frac{1}{6}$-inch (4 mm.) higher power lens replaces the $\frac{2}{3}$-inch lens under the tube. If the two lenses are arranged on the revolver in an absolutely parfocal manner the $\frac{1}{6}$-inch lens, after the turn is made, should be strictly in focus. This, however, is rarely the case, and it is necessary to use the fine adjustment to get a really sharp focus. So the term parfocal has a relative value and meaning only. The term perfectly and identically centred means that the centre of all objectives is in the optical axis of the instrument, so that an object which is exactly in the centre of one objective will also be exactly in the centre of another objective if the latter is swung around to the place of the former by turning the revolver or nosepiece. Here, likewise, instead of absolute correctness only a more or less close approximation is attained. Working with bacteria a $\frac{2}{3}$-inch and a $\frac{1}{12}$-inch objective are generally used. The $\frac{1}{6}$-inch objective, except for the observation of the ray fungus in pus, is generally employed in section work in histology or pathology.

In studying bacterial preparations the $\frac{2}{3}$-inch dry lens is first used for a general orientation of the specimen and to pick out a spot which is neither too much overloaded with bacteria nor overstrained. The supply of organisms, however, should not be scanty nor understained. The oil-immersion lens is now brought into use. It is not advisable to swing around the $\frac{1}{12}$-inch lens and depend upon its being parfocal with the $\frac{2}{3}$-inch lens. Often it is not, and by swinging it around after the low-power objective has been in focus it may strike against the cover-glass or some overhanging margin of the squeezed-out Canada balsam. The glass may damage the expensive high-power lens, and the balsam will certainly soil it and necessitate its being cleaned. It is best to raise the tube before the oil-immersion lens is swung into place. Good modern microscopes for work in bacteriology and hematology are often supplied with an attachable mechanical stage, which permits of a systematic search over the whole of the microscopic preparation. However, instruments for the use of students in their laboratory training are rarely supplied with this desirable accessory. There are also some devices called warm or heated stages which permit the study of bacteria and other microorganisms at higher stationary temperatures.

Stained and Unstained Objects.—An important point in microscopic work which the student should always recollect is the following: When stained objects are examined the colored image shows better the more powerful the illumination. Hence, in the examination of stained preparations the iris diaphragm must be kept wide open.

On the other hand, with unstained bacteria, cells and other small bodies in general, the details of the pictures depend upon the difference in

FIG. 35



A modern student's and practitioner's microscope.

refraction between the different parts of the bacteria (flagellæ, granules, spores, capsules, etc.) and the medium in which they are

suspended (water, bouillon, milk, wine, pus, etc.). These slight differences would be lost in a flood of light and the iris diaphragm should be closed considerably. It is much easier for the beginner to examine stained preparations of bacteria than the unstained ones, hence the steps in the former procedure will be given first.

Steps in Using the Microscope for Stained Bacterial Preparations.—1. Place the instrument in front of the source of light in a vertical (not inclined) position and deposit the preparation on the stage of the microscope. See that the surfaces of the objectives and eye-pieces are free from dust and grease and otherwise clean.

2. Bring the $\frac{2}{3}$-inch low-power lens into the centre and lower the tube on the coarse adjustment so that it is about one inch from the cover-glass.

3. Illuminate the object by manipulating the plane reflector (do not use the concave mirror). Have iris diaphragm wide open and slowly lower the tube on the coarse adjustment until the color of the object can be well recognized. Now, again manipulate the reflector and lower the Abbe condenser until the field of vision is well and uniformly illuminated and until no shadows of the window frame or parts of the lamp are seen in the field of vision.

4. Move the microscopic slide around until a place is found where the stain is neither too dense nor too scanty. Place this spot in the centre of the field and clamp down the slide so that it is immovable. Look again to see whether the selected spot is still exactly in the centre of the field; if not, move the slide under the clamps until the desired spot is where it is wanted.

5. Raise the tube somewhat on the coarse adjustment and swing the $\frac{1}{12}$-inch oil-immersion lens into the centre. Place a drop of cedar immersion oil on the centre of the cover-glass; run the immersion lens on the coarse adjustment into the oil. Raise the tube on the coarse adjustment so that the drop of cedar oil is drawn out. The oil-immersion lens is now considerably above the proper focal distance of the object.

6. Raise the substage condenser as high as it will go. (Its front lens is now on a level with the stage of the microscope.) Again, manipulate the plane mirror so that the field is well and uniformly lighted. Now, slowly, with the coarse adjustment, lower the tube under the guidance of the eye which watches the field of vision through the ocular. As soon as some color is observed the fine adjustment or micrometer screw must be used until a clear image is obtained; that is, until the oil-immersion lens is sharply in focus.

The microscopic picture so obtained may sometimes still be improved by lowering the substage condenser a very little bit, and by closing the iris diaphragm somewhat; but the beginner should be careful in attempting these corrections, which require a good deal of skill and judgment in the interpretation of the image.

The danger for the beginner in the use of the oil-immersion lens

consists in lowering it so much that it is brought beyond the proper focal distance. He is then likely to lower it still farther until the front lens touches the cover-glass and the pressure may be great enough to crack or dislocate the former. The expensive objective is then ruined and can only be repaired at a considerable outlay of money.

FIG. 36

Hollow slide with cover-glass.

Steps in Using the Microscope for Unstained Bacteria in the Hanging Drop or Moist Chamber.[1]—1. The same as step No. 1 for stained preparations.

2. The same as step No. 2 for stained preparations.

3. Illuminate the object—that is, the live bacteria in the hanging drop of water or bouillon—by manipulating the plane reflector (do not use the concave mirror). Have the iris diaphragm closed so that the field is only very dimly lighted. Slowly lower the tube on the coarse adjustment until the margin of the drop can be recognized. Move the margin of the drop near the centre of the field so that the centre of the lens is just over the thinnest portion of the drop. Clamp the slide and see once more whether the desired spot is in the centre of the field. (All this has to be done under guidance of the eye, looking through the low-power dry lens.)

4. Raise the substage condenser to its highest level; raise the tube; place a drop of immersion oil on the cover-glass, which is situated over the concavity of the slide. Now bring the oil-immersion lens into place and lower it until it touches the cedar oil. Keep on lowering the lens on the coarse adjustment, very carefully and slowly, until it firmly touches the cover-glass. This can be ascertained by watching the vaselin which is between the cover-glass and the slide. As soon as the oil-immersion objective presses firmly on the cover-glass resting over the concavity the vaselin will be squeezed out from under it. In so manipulating the immersion lens there is practically no danger of injuring it, because a little excess pressure will crack the cover-glass, due to the poor backing given it by the concavity of the slide. If this occurs the hanging drop will have to be made over again.

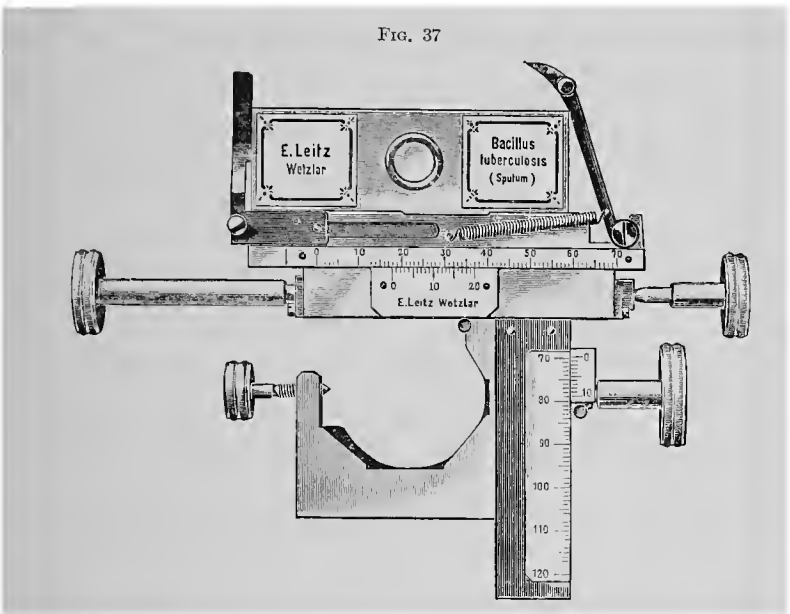
5. After the immersion lens has been lowered as described it is below the proper focal distance. Open the iris diaphragm and see that the field is well and uniformly lighted (if this is the case there is no danger of having an oblique illumination). Now, close the iris diaphragm so that only a small opening is left and the field is very

[1] For steps in preparing moist chamber, see p. 103.

dimly lighted. Raise the tube on the coarse adjustment very slowly and gradually. As soon as the faintest details can be seen, perhaps the margin of the drop or a few individual bacteria, use the fine adjustment to get the exact focus.

In examining both stained and unstained bacterial preparations it is desirable to move the slide in order to examine the whole specimen; this necessitates constant changing of the fine adjustment in order to keep the object in focus.

FIG. 37



Attachable mechanical stage.

Micrometers.—In the study of bacteria their size is always mentioned. The task of measuring so small an object as a bacterium must appear a very formidable affair to the student, yet it is an exceedingly simple procedure. It, however, requires the use of some microscopic accessories, such as a stage micrometer and an eyepiece micrometer. The stage micrometer consists of a glass slide with a finely ruled scale of one millimeter, divided into 100 equal parts, hence the space between two dividing lines is equal to one-hundredth of a millimeter, or $10\mu = 10$ micra $= 10$ micromillimeters. The simplest eyepiece micrometer consists of a circular glass disk with a finely ruled scale which divides a line in the centre of the disk into fifty equal portions. This disk should be laid upon the inner diaphragm of the eyepiece. In order to do this the upper lens of the eyepiece must be unscrewed and then replaced again. Some eyepiece micrometers are very complicated and expensive, but all are made on the same principle, a line in the centre divided into

equal spaces. The steps in the use of the two micrometers for the purpose of measuring bacteria, cells, and other very small microscopic objects are as follows:

1. Unscrew the upper lens of the eyepiece, which exposes the diaphragm of the latter. Place the circular glass disk micrometer, generally held in a small metallic frame, with its flat side downward upon the diaphragm, replace the front lens of the eyepiece and return to its proper position on the draw tube of the microscope. Regulate the upper lens of the eyepiece so that the lines of the eyepiece micrometer show clearly.

2. Place the stage micrometer on the stage and get its scale in focus with the oil-immersion objective. This must be done according to the rules for examining unstained objects, hence the iris diaphragm must be closed as much as possible.

3. The scale of the stage micrometer runs from above downward in the field of vision. Now manipulate the eyepiece micrometer, by rotating the eyepiece, so that the ruled scale of the latter also runs from above downward. Next, move the stage micrometer (this can be more easily done if the microscope has an attachable mechanical stage) so that the first line of its scale and the first line of the eyepiece micrometer fall together (overlap each other).

4. Manipulate the draw-tube so that a number of subdivisions of the eyepiece micrometer are just equal to one partition—that is, the space between two lines—of the stage micrometer.

5. Suppose that eight of the spaces of the eyepiece micrometer are equal to one space of the stage micrometer; then each space of the eyepiece micrometer with the magnification used is equal to 1.25 micra. As already stated, the stage micrometer is so ruled that 1 mm. is divided into 100 equal parts; therefore, each space is equal to 10 micra, and 8 eyepiece spaces = 10 micra; hence, 1 space = 1.25 micra.

6. Raise the tube of the microscope on the coarse adjustment; remove the stage micrometer and replace it by the stained cover-glass preparation of bacteria which are to be measured. Open the iris diaphragm and bring the bacteria into focus.

7. Now manipulate the slide on the stage and the eyepiece by rotating it so that a typical bacterium, say a bacillus, just stands at right angles to the lines of the ruling of the eyepiece micrometer. See that the end of the bacterium apparently just touches one of the lines and note over how many divisions the bacillus extends.

8. Suppose that the bacillus just fills three divisions of the scale; then it is three times 1.25 micron or 3.75 micra long.

9. In making these measurements the following precautions are to be used. The slide on which the bacterial preparation is made should be the same thickness as the stage micrometer slide. After the draw-tube has been adjusted in order to bring a whole number of divisions of the eyepiece into one division of the scale of the stage

micrometer, the draw-tube must be left exactly where it is. If it had been drawn out say to 171.5 millimeters, as shown by its scale, it must be left there after the removal of the stage micrometer and the substitution of the slide with the bacteria. A large number of the most typical forms (not involution forms) must be measured and the size given in terms of the minimum and maximum length, for instance, from 2.5 to 3.75 micra.

Camera Lucida.—The camera lucida drawing apparatus and the photomicrographic camera are important microscopic accessories in the study of bacteria. The use of the former is very simple and explains itself; that of the latter, however, cannot here be taken up in detail, since it requires special studies and much delicate work in order to obtain good photomicrographs. When they are prepared with skill they are by far the best method of illustrating [micro-organisms.

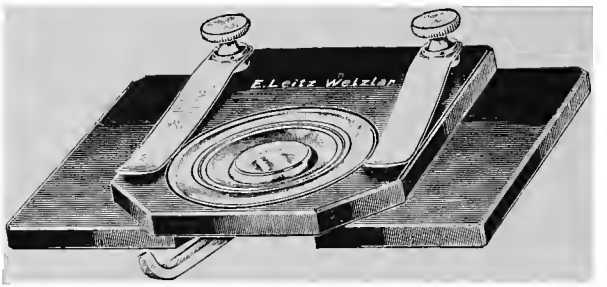
FIG. 38.

Camera lucida drawing apparatus.

Dark-field Illuminator.—A microscopic accessory which has recently come very prominently into use is the dark-field illuminator, devised for observing living bacteria and other microscopic objects. It shows these as the only light objects in an otherwise perfectly dark field of vision. When it is to be used the condenser lens of the sub-stage illuminating apparatus of the microscope must be removed and the dark-field illuminator placed either on top of the stage or slipped into the position that the Abbe condenser lens previously occupied. Instruments are designed to work in either one position or the other, but when constructed for use on top of the stage they cannot be used below it, and vice versa. The dark-field illuminator is so arranged that it will refract the parallel rays of light which are reflected upward by the mirror, so that they will be changed into very oblique rays. These, when they strike the cover-glass of the preparation, suffer what is known as total reflection; that is, they are refracted back in the direction from which they came; in consequence of this the field of

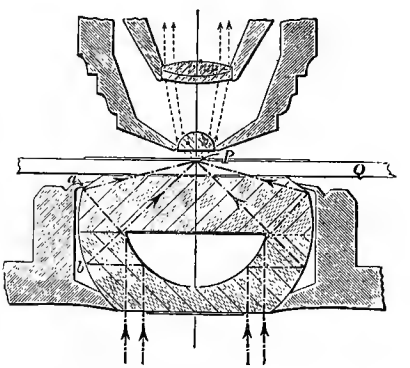
the microscope appears perfectly dark. However, the very central rays of light which strike the cover-glass illuminate the bacteria and

FIG. 39



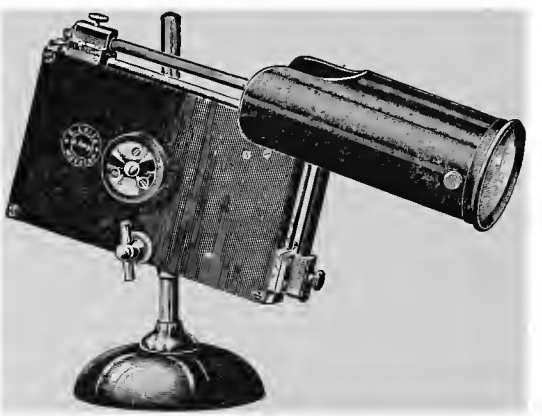
Dark-field illuminator, or reflecting condenser.

FIG. 40



Optical construction of dark-field illuminator.

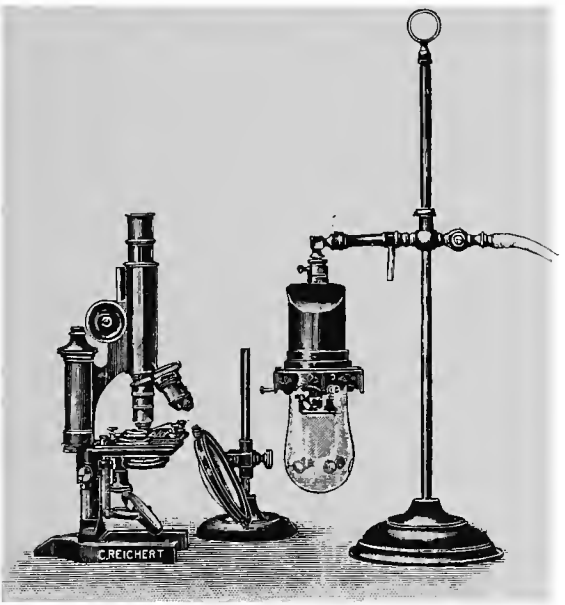
FIG. 41



Electric arc lamp with hand feed for a current of 4 ampères and illuminating lens to be used with the dark-field illuminator.

small particles and these then stand out as very bright objects on an entirely dark background. As a source of light for the dark-field illuminator we can use an inverted Welsbach light, immediately before the reflecting mirror of the microscope; or still better, a small electric-arc light especially constructed for the purpose. Whichever light is used, its rays must be collected by a condensing lens, so that the mirror receives a very strong bundle or pencil of rays of light. The use of the dark-field illuminator is as follows:

FIG. 42



Dark-field illuminator used with Welsbach gas light.

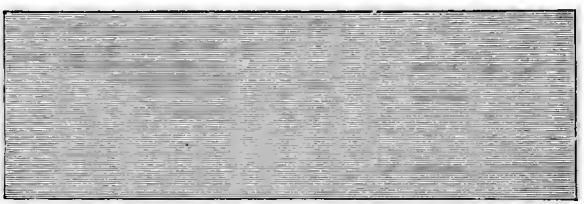
1. Arrange the dark-field illuminator according to the type of apparatus, either on top of the stage or below it.
2. Place a drop of the fluid to be examined on a clean slide and cover with a clean No. 1 cover-glass.
3. Place a drop of immersion oil in the centre of the upper surface of the dark-field illuminator.
4. Place slide on upper surface of dark-field illuminator. There is now no air between the latter and the slide, since these surfaces have the immersion oil between them.
5. Place the instrument so that the light of the inverted Welsbach burner or electric arc light is near the refracting mirror of the substage of the microscope, and place between the source of light and the reflector the concave lens which collects the rays of light. When

an electric-arc lamp is used the condensing lens is combined with it; when a Welsbach burner is used the lens is separate.

6. Manipulate the reflector so that the microscopic field appears uniformly dark, while cells, bacteria, granules, etc., appear as exceedingly light, highly refractive objects.

The dark-field illuminator is not at all difficult to use, and with it fine objects, such as spirochetæ, etc., are found much more easily than with the ordinary hanging-drop or moist-chamber method. The high-power dry lenses $\frac{1}{6}$ and $\frac{1}{8}$-inch focal distance are generally best for use with the dark-field illuminator. There is no great advantage in the use of oil-immersion lenses. If they are to be used at all it is necessary to screw into the interior of the lens a small metal funnel in order to limit the field of vision; this is necessary for optical reasons. It is also necessary to place a drop of immersion oil on the upper surface of the cover-glass in addition to the drop which was placed on the upper surface of the dark-field condenser. The latter is generally supplied with a diaphragm to regulate the amount of light admitted.

FIG. 43



Glass slide.

FIG. 44

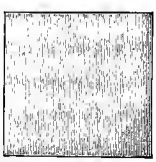
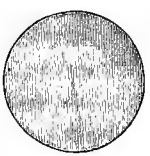


FIG. 45



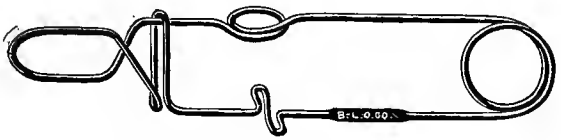
Square and round cover-glass.

Small Utensils.—A number of small appliances and utensils are necessary for the first elementary studies in bacteriology.

Slides and Cover-glasses.—Slides and cover-glasses are used similarly as in normal and pathologic histology. It has previously been pointed out that the cover-glasses or cover-slips for use in work with bacteria should be the thinnest kind, that is, No. 1 (they are from 0.15 to 0.17 mm. in thickness). Both slides and covers should be very clean and free from dirt and grease. As they are generally

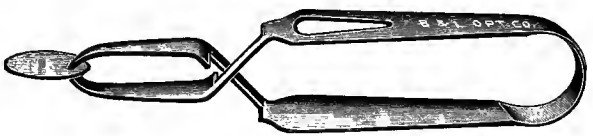
furnished they are free from neither. The best method to cleanse them which will also remove any soluble alkalies adhering to the

FIG. 46



Stewart's cover-glass forceps.

FIG. 47



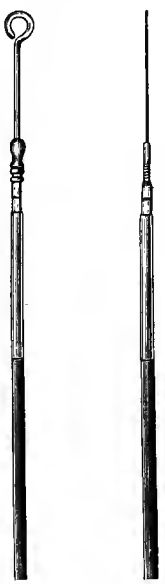
Cornet's cover-glass forceps.

glass, fresh from the factory, is the following: Immerse cover-glasses and slides first in water acidulated with a mineral acid (HCl, HNO_3, H_2SO_4); wash well in ordinary tap water to remove every trace of acid, then wash in alcohol, and wipe dry with a soft clean rag or with Japanese tissue paper.

Forceps.—In order to prepare properly a stained cover-glass specimen it is necessary to hold it in a pair of small forceps. Those used most frequently for work of this kind are the Cornet or Stewart forceps or some of their modifications.

Platinum Rod.—The most important instrument of the bacteriologist is the platinum rod. It consists of a slender glass rod about eight to ten inches long, into one end of which has been fused a piece of platinum wire about twelve to fourteen inches long. For ordinary work the platinum wire is rather thin (No. 26 or No. 27); for special work it is well to have also a strong platinum wire which can easily perforate a tissue. The wire of the platinum rod is either used straight as a needle; or with a round loop on its free end. The latter arrangement is termed the platinum loop and is generally used in cover-glass preparations from pus, other fluids, or pure cultures.

FIG. 48



Platinum needle and loop. For most purposes finer wire is used.

Lamps.—The next utensil required is a small alcohol lamp or Bunsen gas burner. One or the other is necessary for sterilizing the platinum rod, which is never used unless it has previously been heated in a flame and never put aside until it has gone through the same process. This is the only way to avoid contamination of preparations made with outside microörganisms with which the platinum loop has come in contact while in use.

FIG. 49

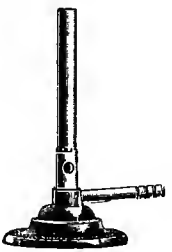
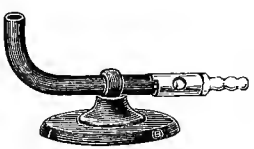


FIG. 50



Bunsen burners.

Glassware.—The dyes used in staining cover-glass preparations are best kept in small bottles provided with droppers. Aside from these, small dishes are needed in which the cover-glass preparations can be washed in water or alcohol. Small beakers or whiskey glasses will do for this purpose. To complete the outfit for elementary work, some small funnels, small round filters, and filter paper are necessary, as it is sometimes desirable to filter the stain directly before use on the cover-glass. A Canada-balsam bottle and some

FIG. 51

Canada-balsam bottle.

FIG. 52

Three staining solution bottles with droppers.

so-called concave slides are also needed. The latter are slides generally a little thicker than ordinary slides, with one or two concavities ground in the glass, and used in making hanging-drop preparations in studying live bacteria.

Method of Preparing a Hanging Drop or Moist Chamber or Concave Slide.—The following are the steps in preparing a hanging drop for studying bacteria and other microörganisms unstained and in the live state:

1. Paint with a camel's-hair brush, around the concavity of a concave slide, a ring of vaselin.

2. Clean cover-glass particularly well, so that it is free from grease; hold in a pair of forceps, and with a platinum loop, place on the centre of the glass a small drop of water, or, better, sterile physiologic salt solution.

3. Enter culture tube with sterile platinum loop and remove from the surface of the agar, gelatin, etc., a small bit of the growth, avoiding at the same time to take any of the culture medium.

4. Rub up the small bit of growth with the drop of water on the cover-glass, so that there is formed a uniform emulsion of the bacteria in water. Spread the drop out considerably so that it is as shallow as possible.

5. As soon as this is accomplished, place the cover-glass over the concavity of the concave slide in such manner that the drop hangs down free into the hollow space.

6. Now press cover-glass down gently into the vaselin surrounding the concavity, so that the latter is closed air-tight.

7. The hanging drop is now ready to be examined in the manner described above.

When bacteria in a fluid excretion like urine, or from a fluid culture medium like nutrient bouillon, are to be examined a drop of these fluids can be placed directly upon the cover-glass without first applying a drop of water.

QUESTIONS.

1. What is meant by a pure culture of a bacterium?
2. What is a colony of a pure culture?
3. At what time in the development of a bacterial growth can colonies best be studied?
4. What properties of bacteria in pure cultures can be recognized without the aid of the microscope?
5. What sources of light are employed in the use of the microscope?
6. What are the main parts of a modern compound microscope adapted for use in work with bacteria?
7. Explain the terms: ocular, objective, revolver, nosepiece, draw-tube, coarse adjustment, micrometer screw, Abbe condenser, iris diaphragm, plane mirror, concave reflector, mechanical stage, oil-immersion lens, dry lens, focal distance.
8. For what distance are the objectives corrected?
9. How far should the draw tube be drawn out and why?
10. What is meant by spherical aberration of an objective? What by chromatic aberration?
11. What is meant by the refraction of light?
12. How is it affected by transparent media of various densities?
13. What is meant by the focus of an objective or its focal distance?
14. In what relation does the focal distance stand to the magnification?
15. Why is a high-power homogeneous immersion lens better than a dry lens of the same focal distance?
16. Explain why very thin cover-glasses are used with the $\frac{1}{12}$ oil-immersion objective?
17. How is the iris diaphragm used when examining stained and unstained bacteria, and why?
18. What does the term parfocal mean?

19. What is meant by exactly and evenly centred objectives?
20. Give in detail the steps in the examination of a stained bacterial cover-glass preparation with the microscope.
21. Give the steps in using the microscope when examining a hanging-drop preparation.
22. How can the size of bacteria be measured?
23. What is (*a*) stage micrometer? (*b*) eyepiece or ocular micrometer?
24. Describe the use of these microscopic accessories in measuring bacteria.
25. What is a camera lucida? What is a photomicrographic camera?
26. What is a dark-field illuminator?
27. How does the microscopic image look when a dark field illuminator is used?
28. Describe the use of the dark-field illuminator.
29. What is a platinum rod?
30. What is a concave slide?
31. What is a Stewart forceps?
32. Describe the method of cleaning slides and cover glasses.
33. Describe the method of preparing a hanging drop in a concave slide.

CHAPTER IX.

STAINING OF BACTERIA IN COVER-GLASS PREPARATIONS AND IN TISSUES.

Anilin Stains.—In laboratory work in histology and pathology eosin is used as a so-called counter-stain; this dye is one of the anilin stains. These stains are complicated bodies derived from anilin oil, a heavy liquid which is, however, not a true oil, but, according to its chemical properties, an alkali or a base, like caustic soda, caustic potash, or ammonia. In fact, it contains the ammonia radical in its molecule. By combining the basic anilin oil with various acids in certain proportions either a *neutral*, an *acid*, or an *alkaline* or *basic salt* may be obtained. Eosin, mentioned above, is an *acid anilin stain.* Bacteria, however, are particularly well stained by *basic or alkaline anilin stains.* The most useful of these for work of this kind are gentian violet, fuchsin, and methylene blue. It is best to keep them on hand in the laboratory in the form of filtered saturated alcoholic solutions, since these do not decompose or deteriorate. Saturated alcoholic solutions contain about 25 grams of the dry stain to 100 c.c. of alcohol. *Watery solutions* are prepared for use from the alcoholic stock solutions by adding to 100 c.c. of distilled water about 5 to 10 c.c. of the saturated alcoholic solution. *It should be remembered that a gentian violet in watery solution stains very rapidly, and easily overstains. Methylene blue stains rather slowly, and therefore does not easily overstain. Fuchsin takes an intermediate position.* Hence, the time for staining is as follows:

With watery gentian violet solution	1½ to 2½ minutes
With watery fuchsin solution	3 to 4 minutes
With watery methylene-blue solution	5 minutes or longer

Staining Bacteria on Cover-glass Preparations.—In staining bacteria with the simple watery anilin stains, say in pus or in any other discharge or excretion, like urine, feces, etc., proceed as follows:

1. A cover-glass which has been thoroughly cleaned is held in a Stewart or similar forceps.

2. Sterilize the platinum loop by holding it over the flame of a Bunsen burner or alcohol lamp. Allow it to cool.

3. Dip the cool platinum loop into the pus, etc., and spread the drop which adheres to the loop in a thin even film on the cover-glass. Sterilize the platinum loop again and put it aside.

4. Allow the cover-glass to become air dry, then, holding it in the forceps, draw it, with the prepared side upward, three times through

the flame. This step is called fixing the cover-glass. By exposing the dried pus, containing the dried bacteria, to the heat of the flame, the proteids (albumins) are coagulated, and the spread, with its pus corpuscles, bacteria, etc., now adheres firmly to the cover-glass.

5. With the cover-glass still held in the forceps, stain for a few minutes with one of the above watery anilin solutions by pouring the stain on the air-dried fixed cover-glass.

6. Wash well in water by moving the cover-glass about in the fluid. Then drop it on filter paper and dry by pressing another piece of filter paper over it. Mount on a slide in Canada balsam and examine first with a low-power lens, then with the $\frac{1}{12}$ inch (2 mm.) homogeneous oil-immersion lens. To mount a preparation, put the Canada balsam on the slide and drop the cover-glass, prepared side down, on the balsam, then press the cover-glass down.

If a bacterial cover-glass preparation from a pure culture is to be examined the first steps in the preparation are as follows:

1. Clean the cover-glass. Hold it in a Stewart forceps, and with a sterile platinum loop place a small drop of water on the centre.

2. Hold an agar or gelatin culture tube in an oblique position between the index and middle fingers of the left hand. Remove the cotton plug and hold it between the middle and fourth fingers of the left hand. Now remove from the open culture tube with the sterile platinum loop a very small amount of the bacterial growth from the surface of the culture medium. Rub up with the platinum loop the growth obtained with the drop of water on the cover-glass, so that an even emulsion of the bacteria is formed.

3. Allow the cover-glass to become air dry. This may be hastened, if desired, by moving it rapidly above the flame. When dry, fix stain, wash, and mount as above.

Precautions in Working with Pathogenic Bacteria.—In working with live, highly pathogenic bacteria, as, for instance, glanders, anthrax, tetanus, etc., the following precautions should be strictly observed in making stained cover-glass preparations:

1. Have on the table, within easy reach of the student, a large china or glass vessel (wooden or non-enamelled metal vessel will not do) filled with a strong solution of bichloride of mercury (corrosive sublimate) at least 1 to 1000, better still stronger.

2. When making the cover-glass preparations be careful not to contaminate anything; sterilize the platinum loop well before laying it down. Never hold the culture tube so that the condensed water or the bouillon can run out and soil the hands, table, cotton plug, or anything else.

3. Pour the stain carefully on the cover-glass, so that none of it runs over. If it did so some dangerous pathogenic bacteria might be washed down on the table.

4. After the stain has acted long enough, pour it into the vessel containing the bichloride solution. In washing a dangerous prepara-

tion it is best to pour some water from a small bottle or a so-called chemical wash bottle over the preparation so that the washing fluid may run directly into the bichloride solution.

5. After being washed, drop the cover-glass from the Stewart forceps on a double layer of filter paper; then sterilize the end of the forceps over the flame of the Bunsen burner. Now put aside the forceps and place a second double layer of filter paper over the cover-glass and dry it by pressing on the paper. Pick up the cover-glass carefully at the margin and move it about a little over the flame to get it perfectly dry. Finally, mount in Canada balsam and throw the filter paper used for drying the specimen into the bichloride solution.

Fig. 53

Wash bottle.

The student should not imagine that staining with watery solutions, air drying, and fixing in the cold kills such bacteria and their spores, as anthrax, tetanus, malignant œdema, etc. He must, therefore, be careful with his cover-glass preparation until it is safely mounted in the Canada balsam. Some bacteria are much more dangerous in the laboratory than out in field practice among patients. The glanders bacillus is one of those which has killed a number of laboratory workers, hence it is particularly necessary to be careful when handling it in pure cultures.

Watery Anilin Stains.—Besides those already mentioned the following watery anilin stains are frequently used:

Loeffler's Alkaline Methylene Blue.—

Saturated alcoholic solution of methylene blue	30 c.c.
Watery solution of caustic potash containing the latter in the very dilute proportion of 1 to 10,000	100 c.c.

This is an excellent all-around dye. It stains cover-glass preparations in from three to five minutes or in even less time; the stain does not decompose easily, and keeps well for many months in a tightly glass-stoppered bottle.

Gram's Method of Staining Bacteria.—This is a very useful method, because it permits the differentiation of certain kinds of bacteria, which are morphologically so much alike that they could not be distinguished merely by microscopic examination. The following solutions are necessary for this stain:

A. Anilin-water Gentian Violet.—Take about nine parts of distilled water and one part of anilin oil. Shake well in a flask or test-tube and filter clear through an ordinary paper filter. Anilin oil is slightly soluble in water. In a saturated solution the excess of the oil shows in oily droplets in the fluid; when filtered these droplets are

arrested by the paper filter, and a clear watery solution with the smell of anilin oil obtained. This watery fluid is known as *anilin water*. Add to the latter enough of a saturated alcoholic solution of gentian violet until a metallic luster is produced on the surface. This indicates that the gentian-violet stain has been added to the point of saturation. The strong stain which has now been prepared is known as *anilin-water gentian violet*. This stain does not keep well and must be made fresh every few days.

B. Gram's Decolorizing Fluid.—

Iodin	1 gram
Iodide of potassium	2 grams
Distilled water	300 c.c.

The steps in staining by Gram's method are as follows:

1. Obtain the cover-glass preparation held in forceps, from pus, a culture, or any other secretion or excretion, as already described. Allow it to become air dry and fix in the flame as usual.

2. Cover the preparation with recently prepared anilin water gentian violet and allow the stain to act for several minutes in the cold, or, better, heat slightly over a flame.

3. Pour off the stain and cover with Gram's iodin decolorizing fluid; change this fluid once, and allow it in all to act one minute.

4. Pour off the iodin solution and wash freely in 95 per cent. alcohol until no more violet color is given off.

5. Allow the alcohol to evaporate or dry between filter paper, and now counter-stain the cover-glass preparation with a weak watery solution of eosin ($\frac{1}{4}$ to $\frac{1}{10}$ watery solution of eosin).

6. Dry between filter paper, mount on a slide in Canada balsam, and examine with oil-immersion lens. Certain bacteria, if treated by this method, appear in a deep violet color, while others appear in a faint eosin (yellowish pink) stain. The bacteria which appear in violet are said to be stained by Gram's method, to hold the Gram stain, or to be Gram positive. Those which are stained faintly pink do not stain by Gram's method, lose Gram's stain, or are Gram negative.

Gram Positive Bacteria.—The following are some of the important pathogenic bacteria which are Gram positive (appear deep violet if stained by Gram's method):

Staphylococcus pyogenes aureus, albus, and citreus.
Streptococcus pyogenes.
Micrococcus tetragenus.
Bacillus tuberculosis.
Bacillus tetani.
Bacillus aërogenes capsulatus.
Pneumococcus.
Bacillus diphtheriæ.
Bacillus anthracis.
Actinomyces.

Gram Negative Bacteria.—The following are some of the important pathogenic bacteria which are Gram negative (appear light pink if stained by Gram's method):

Gonococcus.	Diplococcus meningitidis.
Diplococcus catarrhalis.	Bacillus typhosus.
Spirillum of Asiatic cholera.	Bacillus coli communis.
Spirillum of fowl cholera.	Bacillus of dysentery.
Spirillum of Metchnikoff.	Bacillus of hog cholera.
Spirillum of Finkler and Prior.	Bacillus of influenza.
Bacillus of black-leg.	Bacillus of glanders.
Bacillus necrophorus.	Bacillus pyocyaneus.
Bacillus of malignant edema.	Bacillus mucosus capsulatus.
Bacillus of pneumonia (Friedländer).	Bacillus of bubonic plague.
Spirochetæ of relapsing fever.	

Acid-fast Bacteria.—There is a group of bacteria known as acid-fast bacilli. These take the stain with great difficulty, but hold it even in the presence of dilute acids after they have once been stained. To dye them it is necessary to prepare a very strong staining solution and combine it with a substance which acts as a mordant. This strong staining solution, if used for a short time, must be boiling hot; otherwise, at ordinary or incubator temperatures it must be used for thirty to sixty minutes or longer.

Ziehl's Carbol-fuchsin.—The staining fluid known as Ziehl's carbol-fuchsin is used in staining the following acid-fast bacilli, viz.; the tubercle, leprosy, smegma, Moeller's grass, and Johne's cattle disease bacillus.

Ziehl's Carbol-fuchsin: take

1. Basic fuchsin in crystals	1 gram
2. Absolute alcohol	10 c.c.
3. Water	100 c.c.
4. Carbolic acid (95 per cent.)	5 c.c.

Dissolve No. 1 in No. 2 and No. 4 in No. 3 and mix. (For the use of this stain see under tubercle bacillus.)

Some other special stains are given in the chapters on the different pathogenic bacteria for which they have been particularly devised or for which they are specially valuable.

The following are special methods to bring out differential parts of bacteria, such as capsules, spores, flagella, etc.

Staining of Capsules.—*Johne's Method.*—

1. Stain cover-glass in a warm 2 per cent. solution of gentian violet for one to two minutes.
2. Wash in water.
3. Apply 1 to 2 per cent. watery solution of acetic acid for ten seconds.
4. Wash in water.
5. Examine cover-glass mounted in water—not in Canada balsam.

Friedländer's Method.—

1. Apply to fixed cover-glass preparation a 1 per cent. watery solution of acetic acid for two minutes.
2. Wash in water and dry between filter paper.
3. Stain with anilin-water gentian-violet solution for a few seconds.
4. Wash in water, dry between filter paper, and mount in Canada balsam

Ribbert's Method.—

1. Stain for several minutes in the following solution: Prepare a hot saturated watery solution of dahlia, add to 100 c.c., 50 c.c. alcohol (95 per cent.) and 12.5 c.c. glacial acetic acid.

2. Wash in water, dry between filter paper, and mount in Canada balsam.

Welch's Method.—

1. Prepare and fix cover-glass as usual, then cover the film with glacial acetic acid for a few seconds.

2. Blow off the glacial acetic acid and replace by anilin-water gentian violet. This must be poured on several times to wash off all the acetic acid.

3. Wash in a 1 to 2 per cent. solution of chloride of sodium. Mount in this fluid (not in Canada balsam) and examine.

Staining of Spores.—It has previously been stated that spores, in consequence of the possession of a very tough, tenacious membrane, cannot be stained by the ordinary methods, but require a special technique. The following are some of the methods employed:

1. Prepare and fix cover-glass as usual.

2. Float film on Ziehl's carbol-fuchsin solution contained in a beaker. Place beaker over a small flame or on a water bath and keep the staining solution boiling for twenty to thirty minutes.

3. Wash in water.

4. Decolorize in the following solution: Alcohol, 2 parts; 1 per cent. acetic acid in water, 1 part. Keep on washing until no more red stain is given off. The bacteria are now decolorized, except the spores, which are stained red.

5. Wash in water.

6. Mount cover-glass on a slide in water and examine with a $\frac{1}{6}$-inch dry lens to see whether spores are really stained properly. If this is the case, counter-stain for a few minutes in a weak watery solution of methylene blue.

7. Wash in water, dry between filter paper, mount in Canada balsam. Result of procedure—spores red, remainder of bacilli blue

Moeller's Method.—

1. Prepare and fix cover-glass as usual, then cover it for two minutes with chloroform, which removes fatty matter.

2. Wash in water.

3. Pour on cover-glass a 5 per cent. solution of chromic acid for $1\frac{1}{2}$ to 2 minutes.

4. Stain with watery fuchsin solution heated over a small flame for one minute.

5. Decolorize in 5 per cent. sulphuric acid for five seconds.

6. Wash in water.

7. Counter-stain in dilute watery methylene-blue solution for one-half minute.

8. Wash in water, dry between filter paper, mount in Canada balsam.

Klein's Method.—See chapter on the Anthrax Bacillus.

Staining of Flagella.—Flagella, like spores, cannot be dyed by the ordinary methods, and it is difficult to get a good flagellar stain. It is necessary to prepare the cover-glasses in a special manner. They must first be carefully washed successively in strong mineral acid, water, alcohol, and ether, so that they are absolutely free from dirt, grease, etc. The steps in the preparation of the clean cover-glasses are then as follows:

1. Place six clean cover-glasses in a row, and with the platinum loop place a small drop of water on each.

2. Inoculate the drop on cover-glass No. 1 three times from the margin of the growth of a young agar culture, about eighteen hours old, and not over twenty-four hours old. Mix up the growth well with the water to make a uniform emulsion.

3. Now inoculate drop on cover-glass No. 2 three times from emulsion No. 1 (on cover-glass No. 1), and having made a uniform emulsion on No. 2.

4. Inoculate No. 3 three times from No. 2, and so on until all of the six drops have been inoculated.

5. Allow the six cover-glasses to become air dry, then fix in the following manner: Do not pick up cover-glasses with forceps but hold in the right hand between thumb and index finger, and while in this position fix by passing three times through a flame. In this manner the temperature sense of the worker will prevent an overheating of the cover-glasses and a burning of the delicate flagella.

Treat and stain all six cover-glasses by one of the following methods, and if successful, several good preparations are generally obtained.

Loeffler's Method.—1. Apply to fixed cover-glass the following mordant:[1]

20 per cent. watery solution of tannic acid, prepared by heating. .	100 c.c.
Watery solution of sulphate of iron, saturated in cold. . . .	50 c.c.
Saturated alcoholic solution of fuchsin	10 c.c.

Use the mordant, moderately heated, for one-half to one minute.

2. Remove the mordant and wash well in water.

3. Wash in alcohol.

4. Stain with a warm anilin-water gentian-violet solution to which has been added a trace of a very dilute caustic soda solution.

5. Wash in water, dry between filter paper, and mount in Canada balsam.

Loeffler's method for staining flagella was the first one used and published. It is a difficult one on account of the varying amounts of alkali which must be added to the gentian stain. The following method furnishes better and more uniform results:

[1] The word mordant means a preparation which will so act upon a substance that the latter will take a stain more easily and hold it more firmly—for instance, tannic acid or sulphate of iron are used as mordants in the dyeing of wool in technical establishments.

Bunge's Method.—1. Prepare and fix cover-glasses as described under the Loeffler method.

2. Use the following mordant, which must be several days old:

Concentrated watery solution of tannic acid	75 c.c.
5 per cent. watery solution of liquor ferri sesquichlorati	25 c.c.
Concentrated aqueous solution of fuchsin	10 c.c.

Place mordant in a beaker, float cover-glasses, prepared side down on surface of mordant, heat over a small flame until fluid begins to steam, but do not allow it to boil.

3. Wash well in distilled water.

4. Stain with carbol-gentian-violet solution, which is heated until it steams on the cover-glass.

5. Wash in water, dry, mount in Canada balsam.

Carbol gentian violet is prepared like carbol-fuchsin, viz., take—

Gentian violet	1 gram
Absolute alcohol	10 c.c.
Aquæ dest.	100 c.c.
Carbolic acid	5 c.c.

Carbol fuchsin may be used instead of the carbol gentian violet.

Pitfield's Method.—Prepare the following two solutions, keep them separate, and before use filter and mix.

A. Saturated aqueous solution of alum	100 c.c.
Saturated alcoholic solution gentian violet	10 c.c.
B. Tannic acid	10 grams
Aquæ dest.	100 c.c

Mix in equal proportions before use. Cover film with staining fluid, heat over a small flame until the stain boils, leave on for one minute, wash well in water, dry, and mount in Canada balsam.

The following is not a real staining but a silver impregnation method:

Van Ermengem's Method of Silvering Flagella.—1. Prepare cover-glasses as already indicated, and apply the following mordant, which may be used hot for five minutes or cold for thirty minutes. Take—

20 per cent. watery solution of tannic acid	60 c.c.
2 per cent. watery solution of osmic acid	30 c.c.
Glacial acetic acid	4 to 5 drops

2. Wash in water.

3. Wash in alcohol.

4. Immerse for one to three seconds in $\frac{1}{2}$ to 1 per cent. watery solution of nitrate of silver.

5. Wash for several seconds in the following solution:

Gallic acid	5 grams
Tannic acid	3 grams
Acetate of sodium	10 grams
Distilled water	350 c.c.

6. Immerse again in the $\frac{1}{2}$ to 1 per cent. watery solution of nitrate of silver and move cover-glass, held in forceps, continually until it assumes a black color.

7. Wash well in water, dry, mount in Canada balsam.

Staining the Polar Bodies or Babes-Ernst Granules.—*Neisser's Method.*—1. Prepare and fix cover-glass as usual.

2. Stain with the following solution for one to three seconds:

Methylene blue	1 gram
Absolute alcohol	20 c.c.
Glacial acetic acid .	50 c.c.
Distilled water	1000 c.c.

3. Wash in water.

4. Counter-stain in a 2 per cent. watery solution of Bismarck-brown.

5. Wash in water, dry, and mount in Canada balsam. Result of the stain—polar bodies blue, other parts of bacterium light brown.

Piorkowski's Method.—1. Prepare cover-glass as usual and stain for one-half to one minute in Loeffler's alkaline methylene blue.

2. Decolorize in alcohol containing 3 per cent. hydrochloric acid for five seconds.

3. Wash rapidly in water.

4. Counter-stain in a 1 per cent. watery solution of eosin, very rapidly.

5. Dry between filter paper and mount. Result of the procedure—polar bodies blue, rest of bacterium eosin pink or yellow.

Wright Stain of Eosinate of Methylene Blue.—This stain is an exceedingly useful one, and it has a wide field of application, not so much for staining bacteria from pure cultures, as for bacteria in pus, and particularly for protozoa, such as malarial plasmodia, trypanosomes, piroplasmata, and for blood films in general. It is a modification of the stains of Romanowsky and Leishman. The dye can be bought ready made or can be easily prepared as follows:

1. Prepare in a flask a $\frac{1}{2}$ per cent. of sodium bicarbonate in water. Add, after complete solution, 1 per cent. of methylene-blue in substance (either one of the following three preparations of Gruebler's may be used: methylene-blue BX, Koch's or Ehrlich's rectified).

2. Place the sodium bicarbonate methylene-blue solution in the steam sterilizer, where it is left for one hour after the steam is up. Then remove and allow to cool.

3. Prepare a $\frac{1}{10}$ per cent. watery solution of eosin (yellowish eosin of Gruebler).

4. Place the methylene-blue solution in a large flat dish and add the $\frac{1}{10}$ per cent. eosin solution gradually, stirring constantly with a glass rod. Keep this up until the mixture assumes a purplish color and until a scum with a yellowish metallic lustre forms on the surface and a finely granular black precipitate appears in the suspension. This will generally require about 500 c.c. of the $\frac{1}{10}$ per cent. eosin solution to 100 c.c. of steamed alkaline methylene-blue solution. After the precipitate has formed the fluid is run through a dense paper filter and the precipitate collected. The fluid which runs through the filter is of no further use, and is not kept. The pre-

cipitate on the filter, however, is carefully dried in the incubator or in a drying oven at a low temperature. It is then scraped off the paper filter and from it is prepared a saturated solution in C. P. methylic alcohol (Merck's). Three-tenths of a gram of the dry stain will saturate 100 c.c. of C. P. methylic alcohol. This is the staining fluid, ready for use. It is very permanent in character, provided that it is kept in a tightly glass-stoppered bottle. Care must be taken from the start that the solution does not become diluted with water, hence the bottle in which it is prepared must have been washed out with methylic alcohol (not with water).

The stain also fixes the preparation, and it is used as follows:

1. When thoroughly air dry do *not* draw through flame. Prepare cover-glasses or slides as usual.

2. Pour undiluted stain from glass-stoppered bottle on the cover-glass or slide and allow it to act for one minute.

3. Now add with a dropper, drop by drop, distilled water (distilled water must be used, not ordinary tap or well water) until the mixture becomes semitransparent, with a reddish tint visible at its margins and a metallic scum forms on the surface. The amount of water required will vary with the amount of staining fluid on the preparation, but in general eight or ten drops will be sufficient if a seven-eighths inch square cover-glass is used.

4. Wash in distilled water for about one-half to one minute. This will so differentiate the stain that nuclei and bacteria appear blue, while cell protoplasm appears pinkish. (The stain also differentiates well neutrophilic, eosinophilic, and basophilic granula of the various leukocytes.)

5. Dry between filter paper and mount in Canada balsam.

Giemsa's Stain for Spirochetæ and Protozoa.—This permanent stain is prepared in the following manner: Take

Azur II, eosin (this is a combination stain)	3 grams
Azur II	0.8 grams

Dry in a desiccator over sulphuric acid, powder very fine and sift through a very fine meshed sieve. Dissolve in C. P. glycerin (Merck) 250 c.c. at 60° C. and shake continually. Add 250 c.c. Kahlbaum's C. P. methyl alcohol which has been warmed to 60° C. Keep for twenty-four hours at room temperature and then filter. It is best to buy this stain ready made as prepared by Gruebler, in Leipzig. Use it as follows:

1. The very thin cover-glass or slide preparation must be allowed to become air dry.

2. Fix for fifteen to twenty minutes or longer in absolute alcohol.

3. Dilute the Giemsa stain as follows: Take distilled water and add to each cubic centimeter a few drops of a $\frac{1}{10}$ per cent. solution of carbonate of potassium. To each cubic centimeter of this slightly alkaline watery solution add one drop of the Giemsa stain.

4. Use on fixed cover-glass or slide preparation at once and allow the stain to act not less than one hour, better several hours.

5. Wash well in water, dry carefully between filter paper, and mount in Canada balsam.

If precipitates have been formed on the specimen it is well to wash rapidly a few seconds in 90 per cent. alcohol and then once more stain a short time in the dilute Giemsa stain, without the addition of carbonate of potash, *i. e.*, a drop of the stain to each cubic centimeter of pure distilled water.

Blackening of the Background for Demonstration of Fine Spirochetæ.—Freudenwald has published recently a method of demonstrating a few fine spirochetæ (particularly the Spirochætæ pallida) in secretions which may contain them. The procedure is not a staining method, but one in which a dark background is prepared by the use of Chinese (India) ink, upon which the microörganisms appear as light unstained spiral threads. The method is as follows:

1. Take a platinum loopful of the discharge which is suspected of containing the microörganisms.

2. Mix and rub it up well with a drop of Chinese ink on a clean slide (the author recommends a German preparation fluid Chinese ink of Guenther and Wagner). The mixture assumes a yellowish-brown tint.

3. Spread the drop out into a thin film with the margin of another clean slide and allow it to become air dry.

4. The preparation can now be directly examined with the oil-immersion lens. If the specimen has been well spread in a thin layer, the spirochetæ appear perfectly white on a yellowish or yellowish-brown background. After examination the homogeneous immersion oil can be washed off with xylol and the preparation can be preserved as a permanent one for future use. The author has found this new method very simple and giving very excellent results. Any India ink may be used. It is not necessary to procure the German preparation originally recommended.

Staining Bacteria in Tissues.—It is often desirable and necessary to study the distribution of bacteria in tissues. In order to do this successfully the tissues must first be properly fixed, embedded, and sectioned.

Fixing of Tissues.—Fixing a tissue means its preservation by proper preserving fluids so that its cells and other elements remain as nearly true to nature as possible. Pieces not larger than one cubic centimeter must be cut out with a sharp scalpel or razor. These pieces are dropped at once into strong or absolute alcohol, a formalin solution (1 part of formalin to nine parts of water), or best, if certain stains are to be used, into Zenker's solution.

Bichromate of potassium	2.5 grams
Sulphate of sodium	1.0 gram
Corrosive sublimate	5.0 grams
Water	100 c.c.

Before use add 5 c.c. of glacial acetic acid. The fluid, without the acetic acid, may be made up in bulk, as it keeps indefinitely, but the acid can only be added shortly before use. Leave the tissues in this fluid for from two to twenty-four hours, according to the size of the piece and the hardness or softness of the tissue. Then wash for twenty-four hours in running water. In spite of this washing an insoluble sulphite of mercury will remain in the tissue which must be removed before staining. How this is accomplished with the aid of Gram's or Lugol's iodin solution is described under the steps of the eosin-methylene-blue staining method following.

After tissues have been fixed in a watery fluid, or in 95 per cent. alcohol, they must always be completely dehydrated in absolute alcohol or some other suitable medium.[1] Only after this has been accomplished can the tissues be embedded.

Embedding Methods.—There are two principal embedding methods, and the object of both is to get the tissue into such shape that it can be cut into very thin sections with a razor, or, what is much better, a machine with a special knife called a microtome.

A. Celloidin Embedding Method.—1. After fixation, place the tissue into absolute alcohol for twenty-four hours and change the latter once.

2. Place into equal parts of absolute alcohol and ether for one day.

3. Place in thin celloidin at least for a day, better for several days.

4. Place in thick celloidin at least for a day, better for several days.

5. Paste the piece of tissue with thick celloidin on a block of wood, or, better, on a block of vulcanized wood fiber (a certain wood fiber material impregnated with gutta-percha).

6. Allow the celloidin on the block and on the tissue to become superficially hard; then place block and all in 80 per cent. alcohol. After twenty-four hours the celloidin has hardened well and the tissue can now be sectioned with the microtome. The thin and thick celloidin used in this work are prepared from the solid imported celloidin, which comes in a dark bottle put up with water. It is prepared for use as follows:

(*a*) Drain off the water and remove all traces of it by washing in a little absolute alcohol.

(*b*) Place the dry celloidin into a large glass-stoppered bottle, cutting it up with scissors into small fragments if necessary. Add six to eight ounces of equal parts of absolute alcohol and ether and shake violently until all the celloidin is dissolved. This sometimes takes a couple of hours. The thick syrupy fluid which results after complete solution of the celloidin is the thick celloidin. Take some of this and dilute it with ether to a thin consistency. This is the *thin celloidin.* To get the best results it is necessary to place the tissue finally in some thick celloidin kept in a Stender or Petri dish (see Chapter XII)

[1] Complete dehydration means the complete abstraction of water.

and allow the alcohol and ether to evaporate until the celloidin has become of the consistency of a rather soft Swiss cheese. Then the tissue with a little celloidin around it can be cut out and pasted with a little thick celloidin on the fiber block. After which it is best to leave it under a bell-jar with an open dish of chloroform. In this manner the most homogeneous embedding best adapted for sectioning is obtained. However, it is not necessary in ordinary work to adhere strictly to all of these finer details.

Celloidin embedded material is sectioned on the microtome in such a manner that the microtome knife strikes the tissue very obliquely. Both the knife and tissues should be kept constantly wet with 80 per cent. alcohol. The cut sections must be kept in 80 per cent. alcohol until they can be stained.

PARAFFIN EMBEDDING METHOD.[1]—1. Dehydrate the tissue well in absolute alcohol.

2. Place for several hours in equal parts of absolute alcohol and xylol.

3. Place for several hours into pure xylol.

4. Place for several hours into xylol saturated with soft paraffin.

5. Place for several hours in melted paraffin kept in a suitable paraffin oven at about 54° C.

6. Change the melted paraffin once during this time.

7. Prepare a little paper box or place two lead squares on a glass plate, pour some fresh melted paraffin into the square and place the tissue in it.

8. As soon as the paraffin is superficially hard place the tissue with the surrounding paraffin and leads into the refrigerator or into cold water, so that they are cooled very rapidly. This rapid cooling gives to the paraffin a homogeneous consistency which makes it cut better, and much thinner sections can be prepared. When the tissue and the paraffin have been thoroughly cooled, the excess paraffin is trimmed off, leaving the tissue surrounded by a very small amount of the embedding material. The embedded tissue is then mounted with a little melted paraffin on a block of wood or vulcanized wood fiber. After the mounting is firm, the tissue is ready to be sectioned on the microtome.

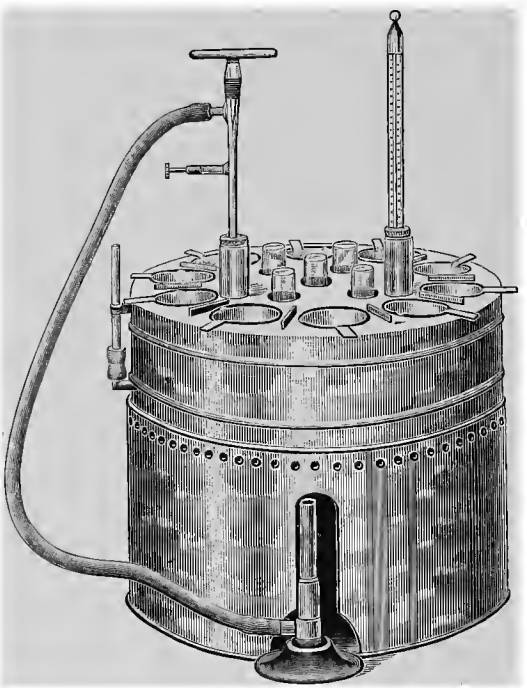
Sectioning.—Paraffin embedded material is sectioned dry with the knife at right angles to the block, and with a rapid stroke. Paraffin sections that are to be stained for the demonstration of bacteria should be very thin—not over five micra—and should lie very flat on the slide. This is accomplished by taking them from the knife,

[1] A very rapid paraffin-embedding procedure is the acetone method, which has the following steps:

1. Place small tissue into best water-free pure acetone for one hour.
2. Change acetone and leave another hour.
3. Drop into melted paraffin and leave in paraffin over one-half hour.
4. Change paraffin and leave another half-hour. The tissue is now ready to be blocked and sectioned.

and floating them in warm water (about 35° to 40° C.), when they flatten out perfectly. The next step is to prepare a clean slide by rubbing over it a small amount of egg-albumen mixture composed of equal parts of beaten white of egg and glycerin, which has been filtered. This egg-albumen glycerin mixture will fix the section on the slide. Dip the slide into the warm water where the section is floating, and guide the latter with a platinum rod or tissue needle onto the slide and move to the part previously prepared with the egg-albumen. After the section is in position the slide is placed for several hours in the incubator to evaporate the water. The section now rests perfectly flat on the dry slide.

FIG. 54



Paraffin-embedding oven.

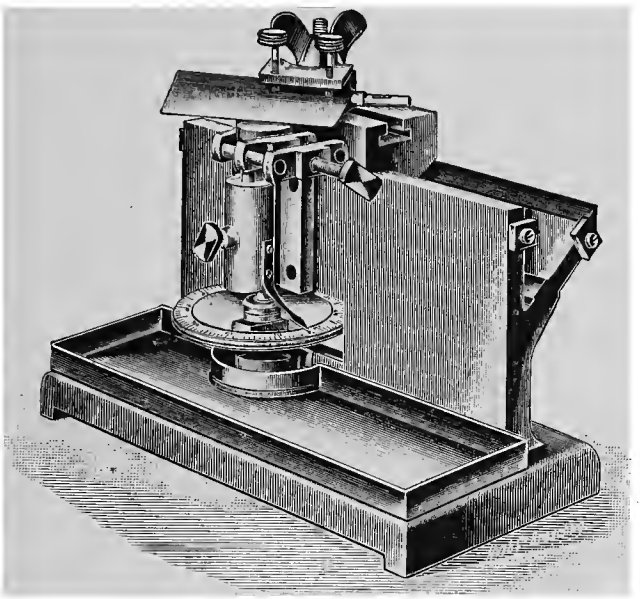
Removal of Paraffin.—It is now necessary to fix the section and remove the paraffin. This is done in the following manner.

1. Move the slide over a flame (alcohol lamp or small flame of Bunsen burner) until the paraffin melts. Then heat a little longer, so that the egg-albumen coagulates and fixes the section on the slide. This manipulation requires some experience which can be gained only by practice. If heated too much the section will be more or less damaged, and if heated too little there is great danger of the

section floating off during the process of staining in the watery solutions.

2. Place the section in xylol which will dissolve out the paraffin.
3. Next, place in alcohol which will wash out the xylol.
4. Remove the alcohol by placing the section in water.

FIG. 55



Small student's microtome for sectioning celloidin or paraffin-embedded tissues.

Staining of Sections.—The section is now ready to be treated by one of the various anilin-staining solutions used to demonstrate bacteria. It is frequently advantageous to first stain the section by some of the methods used in normal or pathological histology, in order to bring out clearly the cellular elements of the tissue itself. If this is done it is generally best to stain the tissue in bulk before it is embedded.

Carmin Stains.—Most useful for such staining in bulk are the carmin stains, particularly alum carmin, which is prepared as follows:

Carmin	2 grams
Alum	5 grams
Water	100 c.c.

Boil twenty minutes, then add enough water to make up for the loss in evaporation. When cool, filter and add a crystal of thymol to prevent the growth of moulds in the staining solution. Tissues to be stained in bulk should be left in this carmin solution for from two to three days; they are then placed for one to two hours in acid alcohol

(alcohol 70 per cent.—100 c.c.—HCl—5 drops), then in several changes of pure alcohol, so that every trace of acid is removed, and finally they are embedded in celloidin or paraffin.

Mallory's Eosin-methylene Blue Stain.—When staining for bacteria in tissues it is frequently necessary to employ different stains according to the species of bacteria to be demonstrated. These stains will, therefore, be given in the chapters on these special bacteria. However, there is one method of staining bacteria known as Mallory's eosin-methylene blue stain, which has a wide range of usefulness and which furnishes excellent results. To get the best results tissues should first be fixed in Zenker's solution. It is necessary to remove from sections so fixed the precipitated mercury sulphite. The steps of the method are the following:

1. After removal of the paraffin and xylol from the sections, place them into Gram's decolorizing fluid (iodine 1 part, iodide of potash 2 parts, water 300 parts) for twenty minutes.

2. Wash out the iodine solution in 95 per cent. alcohol for ten minutes.

3. Wash in water and stain sections for twenty to thirty minutes in a 10 per cent. watery eosin solution.

4. Wash rapidly in water to get rid of the excess of eosin.

5. Stain in Unna's alkaline methylene-blue solution diluted with four to five times its bulk of distilled water for ten to fifteen minutes. Formula for Unna's alkaline methylene-blue solution:

Methylene blue (Koch's)	1 gram
Carbonate of potassium	1 gram
Distilled water	100 c.c.

This solution keeps several months, but it then loses in staining power, because much of the methylene blue is oxidized into methyl violet and methylene red.

6. Wash in water.

7. Wash, for the purpose of decolorizing, in 95 per cent. alcohol, keeping the slide constantly in motion, so that the decolorizing will go on uniformly. When the pink color of the eosin has returned, dehydrate in absolute alcohol and clear in xylol. Then, without mounting in Canada balsam, look at the sections with the low power of the microscope. If the nuclei stand out well differentiated in blue from the eosin-stained protoplasm the section has been decolorized enough. If there is still too much blue present wash in 95 per cent. alcohol again until the differentiation is sufficient.

8. Finally dry and mount in Canada balsam in the usual manner.

Gram's Staining Method for Paraffin Sections.—1. Stain section in warm anilin-water gentian-violet solution for twenty minutes.

2. Wash in normal salt solution.

3. Decolorize for one minute in Gram's decolorizing fluid (iodin 1 part, iodide of potash 2 parts, water 300 parts).

4. Wash in several changes of absolute alcohol until violet color is no longer given off.

5. Clear in xylol and mount in Canada balsam.

Gram-Weigert Method for Celloidin Sections.—In this, as in the preceding paraffin section method, the tissue should have received a preliminary stain in carmin. If not stained in bulk, the sections themselves should be left in the alum carmin over night.

1. Fasten the celloidin section on slide with ether vapor and stain for twenty minutes with anilin-water gentian violet.

2. Wash in normal salt solution.

3. Leave one minute in the iodin solution (solution the same as in preceding method).

4. Wash off in water.

5. Dry with filter paper to remove as much moisture as possible.

6. Wash in several changes of anilin oil to remove most of the voilet stain.

7. Clear with several changes of xylol. Examine with the microscope before mounting in Canada balsam to see whether enough of the violet stain has been removed. If not, wash again in anilin oil, then in xylol.

8. Finally, mount in Canada balsam.

Levaditti Silvering Method for Exhibiting Spirochetæ, particularly Spirochetæ pallida in Tissues in Congenital Syphilis.—This is an impregnation method to impregnate microörganisms in tissues with metallic silver. Although not a staining method, it should be considered here. The steps are as follows:

1. Fix small pieces of tissue in a solution of formalin 1 part, water 3 parts (10 per cent. formalin solution) for twenty-four hours.

2. In 95 per cent. alcohol for twenty-four hours.

3. Wash in distilled water.

4. Place in a 1½ per cent. solution of nitrate of silver in a dark bottle to protect against light; keep in the incubator for three days.

5. Wash in distilled water.

6. Place in the following reducing solution (keep in light and at room temperature):

Pyrogallic acid	2 grams
Formalin	5 c.c.
Distilled water	100 c.c.

7. Wash in distilled water.

8. Embed in celloidin or paraffin.

9. Dehydrate sections, clear in xylol, and without staining mount in Canada balsam.

The spirochetæ appear perfectly black on a yellowish background.

QUESTIONS.

1. What is an anilin stain? What three types are distinguished?
2. Name the three anilin stains most commonly employed in working with bacteria. How are the solutions of these stains prepared?
3. Describe the method of staining a cover-glass preparation of pus for bacteria.
4. Describe method of preparing and staining a cover-glass specimen from a pure culture.
5. What precautionary measure should be observed when staining live pathogenic bacteria?
6. Why is it particularly dangerous to work with live cultures of the glanders bacillus?
7. Give formula for Loeffler's alkaline methylene blue.
8. Give formula for (*a*) anilin-water gentian violet; (*b*) Gram's decolorizing fluid.
9. Describe Gram's method of staining and decolorizing bacteria.
10. Name some bacteria which stain by Gram's method and some which do not.
11. Give formula for Ziehl's carbol-fuchsin.
12. Give Johne's method for staining the capsules of bacteria.
13. Describe one of the methods for staining spores.
14. Give one of the methods for staining flagella.
15. Describe the method of staining the polar bodies.
16. Describe the method of using Wright's stain on a cover-glass preparation of blood or pus.
17. Describe the use of Giemsa's stain for demonstrating Spirochætæ pallida.
18. Describe the celloidin-embedding method.
19. Describe the paraffin-embedding method.
20. Describe Mallory's eosin-methylene-blue staining method.
21. Describe the Gram-Weigert staining method for tissues.
22. Describe Levaditti's silvering method.

NOTE.—The student need not spend much time in learning by heart formulæ for stains and staining methods; these are best acquired by practice.

CHAPTER X.

CULTURE MEDIA AND THEIR STERILIZATION.

A SUCCESSFUL investigation of pathogenic bacteria, permitting of definite, trustworthy conclusions, is possible only if they can be obtained in *pure culture*. By this term is meant the isolation of one species of bacterium to the exclusion of all other living organisms. A pure culture, accordingly, represents an otherwise sterile culture medium containing only one species of microörganisms. To isolate pathogenic bacteria suitable *sterile culture media* are needed. Some of these substances, such as blood serum, milk, potatoes, occur in nature and are simply sterilized by suitable methods without, as a rule, adding anything to them; others are artificially compounded from various substances and chemicals. In the preparation of artificial culture media, the few necessary ingredients must be so selected and mixed in proper proportions that they supply all the elements necessary for the growth and multiplication of bacteria. At the same time the medium must be either neutral or faintly alkaline. Pathogenic bacteria generally grow best on a faintly alkaline medium; a few also grow on a very slightly acid soil, but the latter is rather exceptional. Substances which are changed in a characteristic manner by certain bacteria are frequently added. Sugar, for instance, is introduced to show whether the bacterium which is being grown possesses the faculty of splitting up sugar and forming carbon dioxide. Litmus in an alkaline medium indicates whether the growing bacterium forms acids and finally changes the alkaline reaction to acid.

Natural sterilized culture media, such as milk, coagulated blood serum, potatoes, etc., are not transparent. Very often, however, it is desirable to work with *perfectly transparent culture media*. These can be obtained by adding to suitable clear solutions either *gelatin* or a substance called *agar-agar*, derived from a Japanese sea-weed. Both these substances, when dissolved in a watery fluid by heat, form transparent masses with it. Gelatin culture media which melt at about 25° C. cannot be kept in the incubator without losing their solid consistency. Generally, as stated, culture media must be sterilized. Sometimes, however, it is desirable to use natural media, such as blood, blood serum, ascitic fluid, etc., without subjecting them to heat. In such cases the fluids must be obtained in a perfectly aseptic manner, so that they are and remain sterile.

Sterile blood serum may be obtained from the living animal. Since the method in the case of culture media is identical with that

employed in the collection of an antitoxic or immune serum, it may be here described.

Method of Obtaining Sterile Blood Serum from a Horse.—1. Restrain the horse so that it can be readily manipulated and cannot disturb the operator.

2. Shave a few square inches of skin a little above the middle of the jugular vein.

3. Sterilize the skin by scrubbing well with soap and water, then with alcohol, next with solution (1 to 500 to 1000) of bichlorid of mercury, and finally with sterile distilled water. (An alternate method consists in shaving the skin on the previous day and applying a 10 per cent. alcoholic solution of iodin to the entirely dry skin, half an hour before the operation.) Cover the sterilized skin with sterile cotton.

4. Have ready a large, sterile hypodermic needle or small curved trocar connected with a small rubber tube, leading into a sterile, cotton-stoppered, cylindrical glass vessel. The entire apparatus must be sterile.

5. When the preparations are completed the operator must sterilize his hands as carefully as for an important aseptic operation. An assistant then compresses the jugular vein in the lower portion of the neck. After removal of the sterile cotton from the previously shaved and sterilized skin the trocar is pushed into the jugular vein and the blood flows through it into the sterile receptacle.

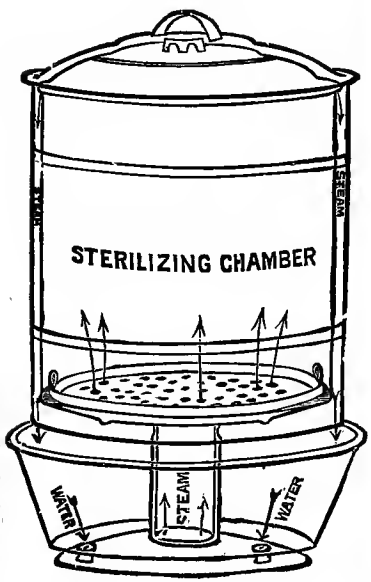
6. When a sufficient quantity has been collected it is placed on ice for twenty-four to forty-eight hours and the serum is then decanted off from the coagulum. Too much stress cannot be laid upon the importance of performing every step in an absolutely aseptic manner.

Sterilization of Bacterial Culture Media.—This is generally accomplished by means of *steam heat*, generated in a suitable vessel called a *steam sterilizer*. In the ordinary apparatus the steam passes out without obstruction; in others it is retained under a pressure of several atmospheres and the temperature rises above 100° C. Those of the latter type are called *autoclaves*. Sterilization is completed in them in a much shorter time (about one hour) than in the common sterilizers with free streaming steam. When culture media are subjected in the steam sterilizer to the temperature of boiling water (100° C.) for a period of three to four hours or longer, all bacteria and their spores are destroyed. This method is called *continuous sterilization*.

Certain media, like blood serum or gelatin, however, are rendered worthless by this method. The former becomes a grumous, broken-up mass, the latter turns into a permanent fluid and refuses to coagulate again. Continuous sterilization in the ordinary steam sterilizer or the autoclave cannot, therefore, be employed in the case of these substances or transudates like ascitic fluid, etc., but short periods of discontinuous heating will answer the purpose. This method is

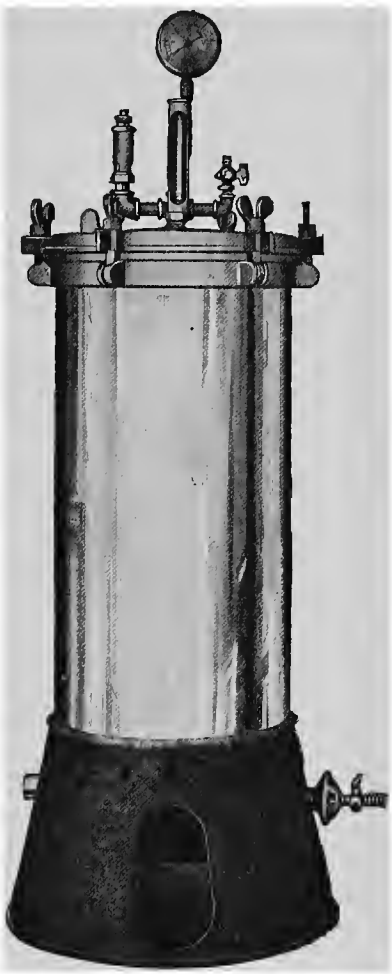
known as the *fractional sterilization of Tyndall.* Its principle is as follows: When culture media are heated for a short time at a higher temperature (for example for thirty minutes at 60° to 100° C.), all adult vegatative forms of bacteria are destroyed. Many spores, however, survive this treatment. If, after having been heated, the medium is left in a warm place for twenty-four hours, most of the remaining live spores will develop into vegetative forms. These are killed by another short exposure to heat. Several repetitions of this process enable all the spores to develop and bring about the destruction of all the bacteria. Culture media containing gelatin are, therefore, sterilized by placing them on three or four consecutive days in the steam sterilizer for periods of from twenty to thirty minutes, and keeping them in the intervals in a warm place.

FIG. 56



Arnold steam sterilizer.

FIG. 57

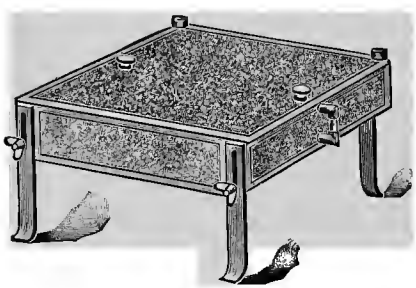


Autoclave, or digester, used for sterilizing under pressure.

Sterilization of Blood Serum or Transudates Containing Blood Plasma.—This is accomplished in the following manner: The fluid is collected

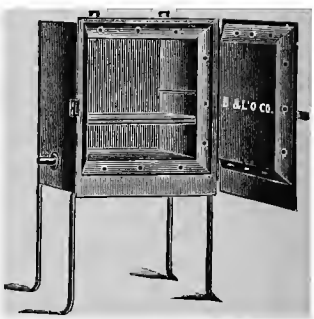
with all possible aseptic precautions and distributed to sterile test-tubes. In these it is heated on a water bath or a special blood-serum sterilizing and coagulating apparatus (Robert Koch) for five to seven consecutive days for one hour at 60° C. During the intervals it is always kept in a warm place. On the seventh or eighth day it is treated as usual and then the temperature is slowly raised to 90° C. and maintained at this height until the serum in the test-tubes is firmly coagulated. The tubes, while in the Koch apparatus, are kept in a slanting position. After removal from the apparatus they are preserved in a cool place until needed. Blood serum may be obtained from sheep, cattle, or swine, at a slaughter house, or it may be drawn directly from the horse, sheep, or goat. In the former case it should be collected in sterile glass receptacles when the bloodvessels of the neck are cut and placed in the refrigerator with as little delay as possible. The less these vessels are shaken the clearer a serum may be expected. After having been on ice for from twenty-four to forty-eight hours the serum has generally well separated from the clot or coagulum. The former may now be poured or pipetted off and distributed into sterile test-tubes or other sterile glass receptacles.

FIG. 58



Blood-serum coagulator.

FIG. 59



Dry-heat sterilizer.

Sterilization of Glassware.—All glassware used for culture media must be sterilized before use. This is best accomplished in a *dry-air sterilizer*. The apparatus is constructed on the principle of a baking oven as found in an ordinary kitchen gas stove or as a detached kitchen utensil. In fact, a baking oven of this kind may be used as a dry sterilizer. In bacteriological work the temperature for dry sterilization may be raised to 160° to 200° C. Because new glassware often contains a slight deposit of soluble alkalies, test-tubes, flasks, etc., must be soaked in water acidulated with hydrochloric, nitric, or sulphuric acid before being used for the first time and afterward washed in pure water until every trace of acid has been removed; otherwise the alkalies would subsequently enter the culture media

and might change their reaction sufficiently to interfere with bacterial growth.

Flasks, test-tubes, etc., before being sterilized in the hot-air sterilizer must be stoppered by plugs of clean cotton. A good surgical cotton gives the best service. Pieces of it are rolled up fairly tightly into a conical plug, which is forced into the mouth, so that some of the cotton projects in mushroom-shape over the rim of the glass. When the test-tubes are to be filled with culture media the cotton plug is removed and afterward replaced. The filled tubes are then sterilized by one of the methods adapted for the particular medium.

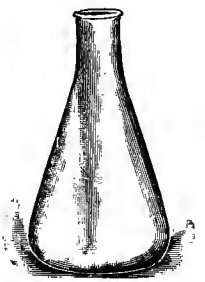
It is, of course, evident that some air will enter the tubes through the cotton after they are taken out of the sterilizer and cooled. Air contains microörganisms, but since it is filtered through the dense mass of cotton all bacteria are efficiently prevented from entering. It is particularly necessary to sterilize all glassware in which blood serum, blood-serum mixtures, gelatin and gelatin mixtures are to be kept, because these media must be sterilized by fractional sterilization and this method is not as safe as a long continuous or autoclave sterilization.

Filling of Test-tubes.—The best method for filling test-tubes with agar or gelatin is as follows: A piece of rubber tubing is connected with the stem of a glass funnel and the free end of the hose is provided with a glass tube drawn out into a narrow outlet; the hose should also have a burette clamp. The melted agar or gelatin is poured *en masse* into the funnel which has previously been warmed with hot water and small amounts can be let out conveniently into each test-tube. A special filling apparatus has also been devised. It is constructed somewhat like a chemical separatory funnel and from it a definite amount of the melted medium (5 or 10 c.c.) can be let out into the test-tubes. In filling test-tubes, it is important not to allow any of the medium to adhere to the mouth of the tube, as this would make the cotton plug stick to the tube.

Protection against Evaporation.—When media distributed in test-tubes are to be kept for some time or are to be placed in the incubator for days and weeks, they must be protected against evaporation. For this purpose *rubber caps* are used. Before being applied to the mouth of the test-tube the caps must be soaked for a number of hours in a solution of bichlorid of mercury (1 to 500 or 1000). This kills the bacteria and moulds which might adhere to the rubber, and by growing through the moist cotton, drop into the culture medium, thus contaminating it. Another method is to seal the mouth of the test-tube with paraffin or sealing wax after the cotton has been pushed down to some extent. Perhaps the *best method of preserving culture media in test-tubes for a longer period of time*, and yet having them always ready and easily accessible, is the following: Take an anatomical jar of the Whitall-Tatum type or an ordinary Mason jar, place some cotton on the bottom, moisten with a strong formalin solution, introduce

the tubes so that they stand in an upright position, moisten the rubber ring with formalin solution and then screw on the lid. Prepared in this manner culture tubes may be kept for many months. Gelatin tubes, however, if kept in this fashion for a long time may lose their property of being liquefied by liquefying bacteria.

FIG. 60



Erlenmeyer flask.

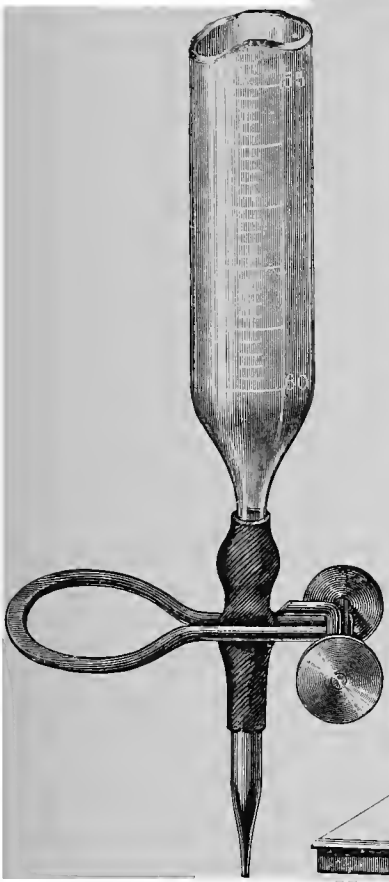
FIG. 61

Pasteur flask.

Preparation of Nutrient Bouillon from Fresh Meat.—The basis of most solid artificial culture media in use is nutrient bouillon. This in itself forms an excellent culture soil for the majority of pathogenic bacteria. On account of the great advantages offered by solid culture media in isolating bacteria in pure cultures, bouillon is frequently combined with agar-agar or gelatin. Nutrient bouillon may be prepared by either one of two methods. Take one pound (500 grams) of finely chopped, lean, boneless meat (generally beef, for special purposes veal, pork, horse or dog meat), add two quarts (1000 c.c.) of water, and allow it to stand for twelve to eighteen hours in the refrigerator or in winter in the cold. Filter through muslin and thoroughly express the juice which remains in the meat. Boil to precipitate the coagulable albumins, then filter and make up to 1000 c.c. Add 10 grams of dried beef peptone and 5 grams of common salt (NaCl) to the filtrate. Boil again until it is entirely dissolved. Test the reaction with litmus paper. It will be found to be acid. Add enough of a solution of sodium hydrate to make the bouillon very slightly alkaline to litmus. Boil again and filter clear. Distribute into test-tubes, about 10 c.c. in each, or small flasks, about 50 to 100 c.c. in each. Sterilize by continuous sterilization in the ordinary steam sterilizer or autoclave. It is sometimes, though not generally, necessary to procure culture media of a very definite reaction. In this case *the bouillon is titrated* with a one-twentieth normal solution of caustic soda or sodium hydrate (NaOH). A small portion of the uncorrected bouillon is taken, diluted with nine times the amount of distilled water, and titrated exactly with the $\frac{n}{20}$ sodium hydrate solution until neutral or faintly acid to a phenolphthalein reaction indicator. The amount of the caustic soda

solution necessary to neutralize 100 c.c. of the bouillon is then calculated and the calculated amount of a normal solution of sodium hydrate, less 1.5 or 1 c.c. is added for each 100 c.c. of the bouillon.

FIG. 62



Mohr's burette and clamp.

FIG. 63

Mohr's burette and holder.

FIG. 64

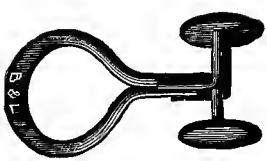
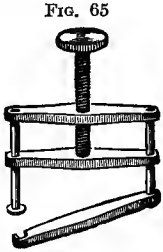


FIG. 65



Burette clamps.

Such a bouillon is designated as a + 1.5 or + 1.0 bouillon. Its reaction is acid toward the phenolphthalein indicator but alkaline toward litmus.

An easier method for preparing the nutrient bouillon is the following: Take 4 to 6 grams of meat extract, 10 grams of dried beef peptone, 5 grams of common salt, dissolve in enough water to make 1000 c.c. Boil well and neutralize with caustic soda solution, then filter clear, distribute to test-tubes, and sterilize as before.

Gelatin.—1. Prepare 1000 c.c. of a clear, faintly alkaline nutrient bouillon. Heat.

2. Dissolve 100 grams of best clear French gelatin in the hot solution. This again makes the solution quite acid.

3. Add caustic soda solution until the mixture becomes faintly alkaline to litmus.

4. Prepare a funnel with a double paper filter through which boiling hot water has been poured. When the latter has drained off, filter the gelatin through the hot filter into a flask. This must be done in a warm room, as otherwise the filter is likely to cool, and the gelatin may set in it. (If necessary, redissolve the gelatin over a water bath, not over an open flame.)

5. While still warm, distribute the filtered gelatin into sterile test-tubes, about 10 c.c. to each.

6. Sterilize by *fractional sterilization,* as already described, for three or four days in the steam sterilizer (not the autoclave). During the intervals keep in a warm place, but the temperature must not be so high as to cause the gelatin to remain fluid.

7. After the last sterilization in the steam sterilizer, keep the tubes in an upright position so that the gelatin sets in a cylindrical mass (not with a slanting surface).

A good gelatin culture media must be perfectly transparent.

Agar-agar.—1. Prepare 1000 c.c. of clear, faintly alkaline, nutrient bouillon.

2. Take 15 to 20 grams of agar-agar and cut the long strips into small pieces, the smaller the better. Soak for from twelve to twenty-four hours in cold water. Drain the water off through a cloth and wring out the swollen mass, so as to remove as much water as possible out of the agar-agar.

3. Add the agar-agar to the bouillon and heat until the former is entirely dissolved. The heating may be carried on in an autoclave, a steam sterilizer, or over an open flame. In the latter case the mixture must be stirred continually so that the agar-agar does not burn.

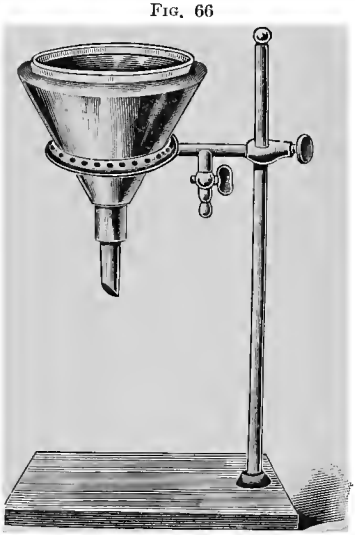
4. When solution is completed, test the reaction again. As a rule it does not change, but remains slightly alkaline, as it was originally.

5. Filter clear. This is the most difficult and tedious process in the preparation of a good agar. Filtration is very slow and sometimes requires a number of days. The process must be carried on in the autoclave, the steam sterilizer, or through a double jacketed copper

filter, which can be filled with water and kept hot over a Bunsen burner (with a ring-shaped burner with many small openings). When it is required on short notice a tolerably good agar can be prepared by sedimentation, as follows: After the agar is entirely dissolved it is placed in cylindrical vessels, which are thoroughly heated in the steam sterilizer and left there when the flame is turned off. During the very slow cooling of the agar the impurities fall to the bottom of the vessel. When entirely cold the agar is caked out and the lower stratum containing the impurities cut off and thrown away.

6. After removal of the impurities by filtration or sedimentation the agar is remelted and distributed into test-tubes, about 10 c.c. to each. It is then sterilized by continuous sterilization in the autoclave or steam sterilizer, and taken out while hot and fluid. The tubes are placed on an inclined plane, so that the agar solidifies in a slanting position. This yields the largest possible surface for inoculation.

FIG. 66



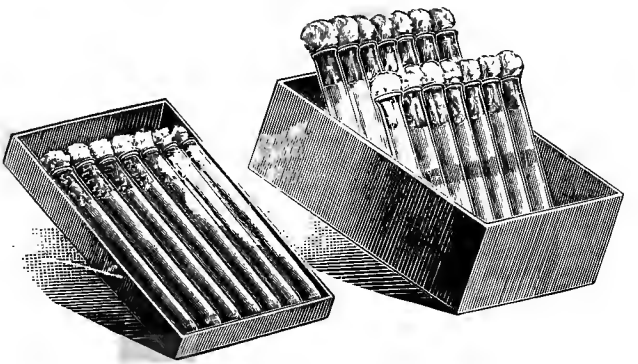
Double-walled hot-water funnel with circular gas burner and stand.

A good 2 per cent. agar, the preparation of which has been described above, should be free from impurities and transparent, but it is never as entirely clear as a first-class gelatin medium. Two per cent. agar melts at 80° C., and resolidifies at 40° C. It can, therefore, be kept in the incubator without melting. Ten per cent. gelatin melting at 25° C. cannot be kept in the incubator. The transparency and brilliancy of both gelatin and agar may be improved by the addition of the white of an egg before filtration. The egg is broken and the white separated from the yolk. The albumen is mixed with water and gradually added under constant stirring to the melted gelatin or agar, the temperature of which, however, must not exceed 60° C. in order to avoid coagulation of the egg-albumen. After thoroughly mixing the media are heated in the steam sterilizer or on a water bath. The egg-albumen coagulates and carries with it to the bottom fine impurities, difficult to filter out without the coagulated albumen.

Both gelatin and agar frequently receive certain additions for special purposes. Such additions may also be made to blood serum or the latter may be combined with agar. The following culture media are frequently used in determining definite characteristics of certain bacteria when raised in pure culture.

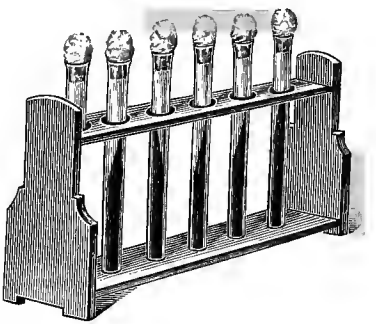
Sugar Gelatin or Sugar Agar.—This is prepared like the ordinary gelatin or agar plus the addition of generally 1 to 2 per cent. glucose, lactose, maltose, saccharose, etc.

FIG. 67



Adjustable copper trays for slanting agar or blood-serum tubes.

FIG. 68



Agar slants ready for use.

Glucose Formate Gelatin (Kitasato).—Ordinary gelatin plus 2 per cent. glucose and 0.4 per cent. of sodium formate.

Litmus Gelatin.—Ordinary nutrient gelatin plus a sufficient quantity of sterile litmus solution to give it a lavender color.

Lactose Litmus Gelatin.—Ordinary nutrient gelatin plus 2 per cent. lactose plus a sufficient quantity of sterile litmus solution to give the transparent culture medium a pale lavender color.

Glycerin Agar.—Prepare nutrient bouillon as usual, but add finally 50 c.c. of pure neutral glycerin for each 1000 c.c.; then treat the agar as described above.

Sugar Agar.—Nutrient agar plus 2 per cent. glucose, lactose, maltose, saccharose, etc.

Lactose Litmus Agar.—Ordinary nutrient agar plus 2 per cent. lactose and a sufficient quantity of litmus solution to give to the medium a pale lavender color.

Gelatin Agar.—This is prepared like the ordinary nutrient gelatin. It contains both gelatin and agar in the following proportions: If it is to be incubated at 30° C., gelatin, 10 per cent.; agar, 0.5 per cent.; if it is to be incubated at 37° C., gelatin, 12 per cent.; agar 0.75 per cent. The gelatin and the agar are dissolved separately in proportionate amounts of the nutrient bouillon. After separate filtration and clearing they are mixed and sterilized by the fractional method, as described for nutrient gelatin.

Loeffler's Blood-serum Mixture.—Prepare an ordinary nutrient bouillon (veal will give better results than beef) and, before correcting the reaction and sterilization, add 1 per cent. glucose. To each 100 c.c. of the sterile bouillon add 300 c.c. of blood serum, distribute the mixture to sterile test-tubes and sterilize according to the method given for blood serum. Coagulate the mixture in a slanting position.

FIG. 69

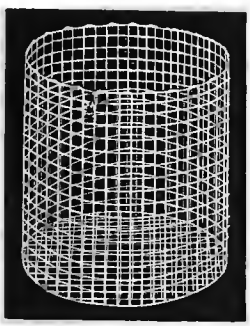
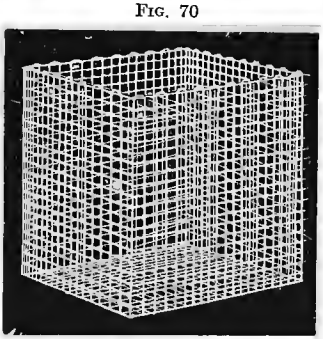


FIG. 70



Wire baskets for agar or gelatin tubes.

Serum Bouillon.—Collect some ascitic, pleuritic, or hydrocele fluid with aseptic precautions in a sterile flask. Mix the serous fluid with twice its bulk of sterile nutrient bouillon. Distribute to sterile test-tubes, sterilize by the fractional, discontinuous method for a week, but do not finally raise the temperature to the coagulation point of albumin.

Blood Agar.—Prepare nutrient agar by the usual method and keep the tubes in the incubator for a few days to permit the condensed water to evaporate. Obtain blood from a rabbit with aseptic precautions by exposing the jugular and drawing it into a sterile all-glass syringe or a glass pipette. Squirt the blood into a small sterile flask containing pieces of glass or glass pearls. Defibrinate the blood by shaking, and pour a small amount of the fluid mixture of blood serum and corpuscles into the dry agar tubes, which can be used at once. In spite of all precautionary measures a certain number of tubes will generally be contaminated. Pigeon's blood is also used.

Hemoglobin Agar.—Prepare agar tubes by the usual method and keep them in the incubator for some time to permit the condensed water to evaporate. Prepare hemoglobin as follows: Allow aseptically obtained blood to run into a flask containing sterile physiologic salt solution. Shake and leave in the refrigerator until the red blood corpuscles have settled. Pipette off the clear fluid and replace by fresh sterile salt solution. Repeat this operation once. (The procedure has been fully described above as the washing of red blood corpuscles). If only a small amount of hemoglobin is needed the washing may be done in the tubes of the centrifuge in fifteen minutes. Remove the hemoglobin from the corpuscles by shaking with ether; evaporate the latter on a water bath at a low temperature or in the incubator, and filter the watery solution of hemoglobin through a Pasteur filter. Add the clear hemoglobin filtrate to dry sterile agar tubes.

Milk.—Milk is sometimes used as a culture medium. It is first boiled, the cream is removed after it has separated out, and 10 c.c. of the fat-free milk is placed into test-tubes. These are then sterilized as usual. Before the tubes are prepared the reaction of the milk must be tested; it should be + 10 to + 20 acid to phenolphthalein, but neutral or faintly alkaline to litmus.

Litmus Milk.—Prepare the milk as above and before distributing it to tubes add enough sterile litmus solution to give it a deep lavender color. If the milk is too acid to produce this color effect, add enough $\frac{n}{20}$ solution of caustic soda to produce the desired color.

Beer Wort.—Wort gelatin and wort agar are frequently used for the cultivation of yeast cells, saccharomyces, or blastomyces. Beer wort, before hops have been added, may be obtained from a brewery. It can also be prepared in a laboratory as follows:

1. Take 250 grams of crushed malt and place it in a 2 liter flask.
2. Add 1000 c.c. distilled water heated to 70° C. and close the flask with a rubber stopper.
3. Place in a water bath kept at 60° C. for one hour.
4. Strain through muslin into another flask and heat in the steam sterilizer for one-half hour.
5. Filter through a dense paper filter and sterilize in the steam sterilizer. If the beer wort is to be used as such it should be distributed to the test-tubes before the sterilization.

Wort Gelatin and Wort Agar.—These are prepared like ordinary nutrient gelatin or agar, except that the nutrient bouillon is replaced by beer wort. The reaction of the latter is not interfered with; however, if gelatin is used the strongly acid reaction of the latter has to be corrected.

Potato Culture Media.—For ordinary work the preparation is as follows: Select good potatoes which have not yet germinated in the cellar; potatoes which have been frozen cannot be used. Their outside must be thoroughly cleansed by scrubbing with soap and water, then with pure water, and finally with 1 to 1000 bichloride solution.

This operation is necessary because earth bacteria often adhere to the outer surface and it is desirable to get rid of their exceedingly resistant spores which can withstand steam sterilization for more than four hours. Cut out the eyes, and, after peeling, cut the potatoes into slices, subsequently to be placed in Petri dishes (see Chapter XII) and into cylinders which are divided into two equal masses to be placed in test-tubes. Cylinders are prepared with an ordinary household apple corer or with a special device, the Ravenel potato cutter. The slices and cylinders are then best washed in running water for several hours to insure partly against subsequent undesirable changes. After washing, the material is placed into the glass receptacles. The tubes first receive a piece of broken glass or some glass wool on which the half cylinder rests, and a little distilled water to supply moisture. The potatoes then undergo fractional or continuous sterilization. In spite of all precautions, potato media frequently dry out and become dark during preservation or later in the incubator. Special points in the use of potatoes are mentioned under anthrax and glanders (see Chapters on these Bacteria).

Special Culture Media.—Certain growing bacteria have definite biologic characteristics, such as the production of indol, etc., and a number of special culture media are used to determine these. Other special media are free from albumins or proteid matter, and contain chemicals of well-known formulæ only. Some media of this type are:

Dunham's Peptone Water.—1. Witte's dry peptone, 10 grams; common salt, 5 grams; mix with 250 c.c. of distilled water, heated to 60° C.

2. Place into a flask and make up with distilled water to 1000 c.c.
3. Heat to boiling for one-half hour.
4. Cool and filter.
5. Distribute to test-tubes and sterilize in the steam sterilizer for twenty minutes on three consecutive days.

Peptone Rosolic Acid Water.—1. Take Dunham's peptone water, 100 c.c., and add 2 c.c. of $\frac{1}{2}$ per cent. alcoholic solution of rosolic acid (coralline).

2. Heat for one-half hour.
3. Filter through filter paper.
4. Distribute to test-tubes and sterilize as above.

Nitrate Water.—1. Dissolve 10 grams of peptone in 1000 c.c. of ammonia-free distilled water.

2. Heat in steam sterilizer for twenty minutes.
3. Add 1 gram of sodium nitrite (C. P.).
4. Filter, distribute to tubes, and sterilize as above.

Albumin-free Solutions.—Pasteur's Solution.

Ammonium tartrate	1 part
Saccharose or cane sugar	10 parts
Yeast-ash	1 part
Water	100 parts

Cohn's Solution.

Phosphate of potash	0.10 gram
Crystals of sulphate of magnesium	0.10 gram
Tribasic phosphate of calcium	0.01 gram
Ammonium tartrate	0.20 gram
Aq. dest.	20.00 c.c.

Ushinsky's Solution.

Chloride of sodium	5 to 7 grams
Chloride of calcium	0.1 gram
Sulphate of magnesium	0.2 to 0.4 gram
Dipotassium phosphate	2 to 2.5 grams
Ammonium lactate (acid)	6.7 gram
Asparaginate of sodium	3.5 gram

Dissolve in 1000 c.c. of distilled water and add 30 to 40 c.c. of glycerin. Sterilize by the discontinuous method.

Fraenkel's Solution.

Chloride of sodium	5 grams
Potassium diphosphate	2 grams
Ammonium lactate (neutral)	6 grams
Commercial asparagin	4 grams
Aq. dest.	1000 c.c.

Add enough caustic soda solution to make the reaction faintly alkaline, distribute to tubes and sterilize by the discontinuous method.

Winogradsky's Solution for Nitrifying Microörganisms.

Sulphate of ammonium	1 gram
Sulphate of potassium	1 gram

Dissolve in distilled water 1000 c.c. and add a previously sterilized solution of magnesium carbonate. Distribute to tubes or flasks and sterilize for three days by fractional method for periods of twenty minutes each.

Winogradsky's Culture Medium for Bacteria which Oxidize Nitrites into Nitrates.

Sodium nitrite, C. P.	1.0 gram
Potassium phosphate	0.5 gram
Magnesium sulphate	0.3 gram
Carbonate of soda (dry)	1.0 gram
Chloride of sodium	0.5 gram
Sulphate of iron	0.4 gram
Distilled water	1000 c.c.

This fluid also forms the base of a 1.5 per cent. agar medium.

Nitrate Solution.

Sodium chloride, C. P.	0.5 gram
Dry peptone	1.0 gram
Potassium nitrate, C. P.	0.2 gram
Distilled water	1000 c.c

QUESTIONS.

1. What do we mean by the term pure culture?
2. What is a culture medium?
3. What is a natural, what an artificial culture medium?
4. What are the requirements of a culture medium on which bacteria can be grown? What should be the reaction of a culture medium?
5. What substances are used to prepare solid transparent culture media?
6. At what temperatures do the ordinary gelatin and agar culture media melt?
7. Describe the method of obtaining sterile blood serum from a horse.
8. How are the artificial culture media generally sterilized?
9. What is meant by the term sterilization?
10. What is an Arnold steam sterilizer?
11. What is an autoclave?
12. What is meant by continuous sterilization?
13. What is Tyndall's fractional sterilization method? What is its underlying principle?
14. How is agar, how is gelatin sterilized, and why?
15. How is blood serum in tubes sterilized and why?
16. How is the blood for use as a bacterial culture medium generally obtained and how treated afterward?
17. How is the glassware used for work in bacteriology sterilized?
18. How should new glassware be treated before use in work with bacteria and why?
19. Give the formula for the preparation of the ordinary nutrient bouillon.
20. What is meant by a + 1.5 bouillon? How does it act toward the phenolphthalein, how toward the litmus indicator?
21. What is the meaning of the word indicator as used in chemistry?
22. What is the meaning of the word normal, or standard, or molecular solution in chemistry?
23. How is a $\frac{n}{10}$ (one-tenth) normal solution of NaHO (caustic soda) prepared?
24. Give the formula for preparing the ordinary gelatin as used in the bacteriologic laboratory.
25. Give the same formula for agar.
26. How is agar filtered or cleared?
27. Name some substances frequently added to the ordinary culture media. Why added?
28. What is a glucose gelatin, what a lactose litmus gelatin, what a glycerin agar, what a litmus milk?
29. How is Loeffler's blood serum mixture prepared?

N. B.—The student should only attempt to commit to memory the formulæ for the most commonly used artificial culture media, such as ordinary gelatin and agar and those indicated in questions 28 and 29. It is unnecessary to commit to memory all of the formulæ given above.

CHAPTER XI.

CULTURE MEDIA IN RELATION TO METABOLIC PRODUCTS—TESTS FOR THE LATTER.

A CONSIDERABLE number of the culture media described in the preceding chapter are especially devised in order to demonstrate the formation of certain metabolic products of bacterial growth, which in some instances are so characteristic that they assist materially in the identification of certain species of bacteria.

Proteolytic Ferments.—As already pointed out, it is often important to determine whether bacteria produce a peptonizing, proteolytic ferment. This can be ascertained by growing the culture on the boiled white of an egg, blood serum, or gelatin. Since the liquefaction of gelatin is the most complete, it is the preferable medium for the identification of peptonizing species. The presence of other ferments, such as diastase, invertin, etc, sometimes has to be determined.

Diastase.—The enzyme diastase can be demonstrated by the addition of starch to a fluid culture medium which has been made absolutely sugar-free. To remove the traces of sugar contained in both meat and meat extract the former after having been finely chopped is kept for two days at 10° to 15° C. At this temperature the muscle sugar is decomposed into lactic acid. According to Theobald Smith, the meat of poorly nourished tubercular cattle is also free from sugar. A *sugar-free bouillon* can also be prepared by inoculating nutrient bouillon with colon bacilli (which decompose the sugar) and subsequent sterilization and filtration of the medium.

After a sugar-free bouillon has been obtained, from 1 to 2 per cent. of the best acid-free starch is added and gelatinized. The process is as follows: Shake together 200 c.c. sugar-free bouillon and 10 to 20 grams of starch; heat 800 c.c. of bouillon to the boiling point and add gradually the 200 c.c. of starch emulsion. Stir continually to insure a uniform gelatinizing of the starch, distribute to test-tubes and flasks and sterilize by the fractional method. Long-continued sterilization might form sugar from the starch. After sterilization some of the tubes and flasks are tested for sugar, and if free from it the media may be inoculated. If a *diastase* or *amylolytic ferment* is liberated in the bacterial growth some of the starch will be converted into *amylodextrin, achroödextrin,* and *maltose.* The presence of these bodies is indicated (1) by a liquefaction of the gelatinized starch, (2) by the iodin test, (3) by testing the reducing power of the fluid with Fehling's solution, the well-known reagent determining

the presence of sugars. The iodin test is made by adding and mixing successively a few drops of Gram's iodine solution with the media. Amylodextrin and erythrodextrin, if present, produce a purple color; erythrodextrin and achroödextrin, a port-wine color; achroödextrin and maltose, no coloration. The qualitative test for maltose is made with Fehling's solution in the usual manner: Take a few cubic centimeters of Fehling's solution, prepared from equal amounts of solution No. 1 and No. 2, dilute with distilled water, heat to boiling, and then add, drop by drop, some of the previously diluted and filtered starch culture medium. If maltose is present an orange or red-brown precipitate will be formed. If the sugar formed is to be determined quantitatively it must first be ascertained whether it is maltose or dextrose. This can be done by changing the sugar present into an ozazon by the action of phenylhydrazin hydrochlorate and acetate of potash and determining the form of crystallization, the melting point, and the amount of nitrogen present in the compound. These are somewhat more complicated, though not difficult, chemical manipulations, the details of which can be found in a text-book on organic quantitative analyses. After the kind of sugar present has been found the amount can also be ascertained by titrating with Fehling's solution.

Invertin.—To demonstrate the formation of invertin in a bacterial growth the latter must be inoculated into a sugar-free bouillon to which a small amount of pure saccharose or cane sugar, which does not possess any reducing power, finally has been added. The presence of invertase is manifested by the inversion of some or all of the cane sugar into invert sugar (dextrose, maltose, etc.), which reduces Fehling's solution.

Rennet and "Lab" Enzymes.—The presence of rennet and "lab" *enzymes* is ascertained in the following manner: Prepare a sugar-free bouillon and inoculate several tubes with the bacterium to be tested. After keeping the growth a few days in the incubator, heat the tubes for thirty minutes in a water bath at 55° C. This will destroy the bacteria but not the rennet if any is present. After heating, add about 5 c.c. of the contents of these tubes to sterile litmus milk in another set of tubes. Keep for several days at 22° C., and examine every day for ten or twelve days. If there is no coagulation at the end of this time rennet is not present.

Acid Formation and Alcohol.—Acid formation by bacteria is accurately determined by inoculating them into 500 to 1000 c.c. of sterile sugar bouillon (see above) of a known definite slightly alkaline reaction. After a number of days the reaction of the inoculated bouillon is again titrated by the aid of a one-twentieth normal solution of caustic soda. The difference in the results of the two estimations indicates the amount of acid formed. If the growth in the flask is very heavy it may be necessary to filter the bouillon through a Pasteur or Berkefeld filter before the final tests can be made.

If alcohol has been formed from the sugar it will be necessary to obtain it by fractional distillation. The details of exact acid and alcohol determinations require a more complicated apparatus and extensive set of reagents, hence they cannot be given here. The mere fact of acid formation can be easily demonstrated on lactose litmus gelatin or litmus milk. If enough acid is produced the lavender color of these media is changed to red.

Gas Production.—Gas production is controlled by inoculating sugar bouillon contained in a fermentation tube. Various kinds of sugars, such as saccharose, dextrose, maltose, lactose, mannite, are used, as it is often more important to determine the varieties of sugar which are or are not split up by certain species of bacteria. The closed limb of the fermentation tube should be graduated so that the amount of gas formed can be estimated approximately from day to day by the readings. After the formation of gas has ceased the proportion of carbon dioxid and hydrogen present can be approximately estimated by the following method: Fill the open bulb of the tube completely with a 2 per cent. caustic soda solution. Close the bulb with the thumb or a rubber stopper and invert the tube several times so that the gas is intimately mixed with the fluid, which now contains caustic soda. The latter will absorb the carbon dioxid. Return the remainder of the gas to the closed end of the tube, remove the thumb or stopper and allow the fluid to cool so that the residual gas is no longer expanded by heat. The loss in gas represents the carbon dioxid and the balance is hydrogen. The proportion of carbon dioxid to hydrogen is often characteristic for certain bacteria.

FIG. 71

Fermentation tube.

Sulphuretted Hydrogen.—To ascertain the formation of sulphuretted hydrogen a special culture medium known as iron peptone or lead peptone is necessary. These culture media are prepared as follows:

1. Take peptone, 30 grams, shake with water heated to 60° C.
2. Wash emulsion into a liter flask with 80 c.c. of water.
3. Add chloride of sodium, 5 grams, and phosphate of sodium, 3 grams, and make up to 1000 c.c.
4. Heat for thirty minutes in a water bath or steam sterilizer to dissolve completely, then filter clear.
5. Fill into tubes 10 c.c. to each and add 0.1 c.c. of a 2 per cent. neutral solution of tartrate of iron. This causes a yellowish-white precipitate to form.
6. Sterilize for twenty minutes on three consecutive days.

The lead peptone is prepared in the same manner except that 0.1 c.c. of a 1 per cent. neutral solution of lead acetate replaces the tartrate of iron added to each tube. The tubes are inoculated with the

bacterium to be investigated and kept in the incubator, together with non-inoculated control tubes. If hydrogen sulphide is formed, a brownish-black to black precipitate appears.

Ammonia.—The detection of ammonia is more complicated. It requires distillation after the culture has been grown in several hundred cubic centimeters of the culture medium, and the media, of course, must be prepared with ammonia-free reagents, including ammonia-free water. The distillate is tested with Nessler's reagent, which gives a yellow color in the presence of free ammonia. The intensity of the color depends upon the amount of ammonia formed, and the test can be arranged as a colorimetric quantitative method.

Indol.—Indol is not infrequently produced in the growth of certain bacteria. These must be inoculated into Dunham's peptone water (see above) kept in tubes which have been incubated for several days. Before making the test the tubes are cooled in running water and then the following reagents are added: (1) A few drops of a 1 to 10,000 sodium nitrite watery solution; (2) chemically pure sulphuric acid, drop by drop, until about 1 c.c. has been added to the 10 c.c. contained in the culture tube. Red discoloration indicates the presence of indol. If a nitrite has been formed in the growth the red color will appear on the addition of the pure strong sulphuric acid alone.

Phenol, or Carbolic Acid.—Some bacteria also produce phenol, or carbolic acid. To ascertain its formation about 100 c.c. of a nutrient bouillon are inoculated. After growth has continued for several days, approximately one-fifth to one-sixth of the fluid is distilled over. The distillate contains the carbolic acid. It is tested by adding first a few drops of lactic acid and then gradually a dilute solution of the sesquichloride of iron. If phenol is present an amethyst-violet color is produced.

Nitrite Formation.—Some bacteria possess the power to reduce nitrates to nitrites. This characteristic can be ascertained by inoculating the nitrate solution described in the preceding chapter with such bacteria. The cultures are best kept for one week at a temperature of 28° C. Two solutions are necessary for the nitrite test:

1. Sulphanilic acid 0.5 gram, dissolved in 150 c.c. acetic acid of specific gravity 1.04.

2. Amidonaphthalin acetate 0.1 gram, boiled in 20 c.c. distilled water, then filtered through cotton and diluted with 180 c.c. dilute acetic acid.

Before use, mix equal quantities of 1 and 2, and add 2 c.c. of the mixture to 3 c.c. of the culture. A red color indicates the formation of nitrites, and its intensity corresponds to the smaller or greater amount of nitrites present. In this, as in other similar tests, control tests must be made with non-inoculated tubes, which are kept in the incubator for the same length of time.

QUESTIONS.

1. How can it be shown whether bacteria in their growth produce a proteolytic ferment or not?
2. What other enzymes are produced by bacteria in their growth?
3. What is an enzyme?
4. Describe the preparation of culture media containing starch.
5. Describe the preparation of a bouillon absolutely free from sugar.
6. Why is the agar medium not adapted for preparing a sugar-free culture soil?
7. What is diastase?
8. What is its effect upon starch?
9. What test can be used to demonstrate the presence of amylodextrin, erythrodextrin, and achroödextrin? What are the color reactions of this test?
10. What sugar is formed by the action of diastase upon starch?
11. What is meant by an amylolytic ferment?
12. What is Fehling's solution? What reaction occurs if sugar is boiled with it?
13. What is the meaning of the statement that sugar is a reducing substance?
14. Describe the determination of the special kind of sugar present in a solution.
15. What are rennet and "lab" enzymes? How can they be detected in bacterial growth?
16. How is the acid production of bacteria demonstrated? How is the amount of acid formed determined accurately?
17. How can alcohol formation be detected?
18. What apparatus is necessary to determine the formation of gas in bacterial cultures?
19. Describe the test to determine whether part of the gas formed is carbon dioxide.
20. What other gas is usually formed with carbon dioxide from sugar?
21. What culture media are used to determine whether hydrogen sulphide is formed? What reaction shows the formation of this gas?
22. What reagent is used to detect the formation of Ammonia?
23. What is indol? What culture medium is used to demonstrate its formation?
24. Describe the test for indol.
25. Describe the test for the detection of carbolic acid.
26. What kind of a process is the change of nitrates to nitrites?
27. What culture medium is used to demonstrate the formation of nitrites?
28. Test for nitrites?

CHAPTER XII.

METHODS OF OBTAINING PURE CULTURES FROM PATHOLOGIC MATERIAL AND OTHER SOURCES.

After the bacteriologist is in possession of various types of sterile culture media he may undertake the preparation of pure cultures from pathologic lesions due to pathogenic bacteria or from other materials, such as milk, water, food, etc. In some cases this is a very simple and easy task. For example, if an animal has a deep-seated abscess which has not broken by ulceration and the bacterial cause is to be determined the procedure would be as follows: Have in readiness a number of culture tubes, some slides, a platinum loop, an alcohol lamp, several knives, and what is required to sterilize the skin.

1. An assistant shaves and cleanses the skin with soap and water, strong alcohol, solution of corrosive sublimate 1 to 500 to 1000. The skin is then dried with sterile cotton and covered with the same material.

2. The knife is heated over an alcohol flame, unless it is kept in a small portable instrument sterilizer. After the removal of the sterile cotton an incision is made which opens up the abscess.

3. In the meantime the one who is to obtain the pure culture has prepared himself as follows: He holds two culture tubes in his left hand. Their upper ends have previously been heated over the flame, which destroyed all bacteria that may have collected externally on the cotton. When everything is ready the cotton plugs are removed by a rotary twist and held between the second and third or third and fourth fingers of the left hand. The tubes must be held obliquely, not vertical, because in this position bacteria from the air may fall into them, nor horizontally, because the condensed water would then run out.

4. The platinum loop is heated over the flame, allowed to cool for a few seconds, and dipped into the pus in the abscess cavity and then introduced into the first tube. There it is dipped into the condensed water and rubbed over the slanting surface of the culture medium. This procedure is called *inoculating* the culture tube.

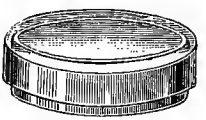
5. After the first tube has been inoculated the platinum loop is again sterilized and a second tube is inoculated like the first one.

6. The platinum loop is again sterilized and laid aside and the right hand, now free, closes the two inoculated tubes with the cotton plugs; these are again burned superficially over the flame (this is called *flamed*) and set aside.

7. The platinum loop is once again brought into use to make some smears on the slides or cover-glasses which have been in readiness for this purpose. When working around animals it is generally better to use slides, because they can be handled more easily than the fine delicate cover-slips, and the subsequent preparation by air drying, fixing, and staining is the same for either. If only one species of bacterium were present in the abscess and the work of cleansing the surface and opening the abscess cavity was aseptically performed, pure cultures should be obtained in the two test-tubes inoculated.

Plates and Petri Dishes.—The procedure just described will give results, but a much better method is to prepare plates. This was first practised by Robert Koch, but his method was somewhat complicated and easily leads to contamination, hence it has been largely replaced by the use of the Petri dish. The latter is a small, circular glass vessel about four to six inches in diameter, one-half inch high, and provided with a cover. These Petri dishes must be sterilized before use in the hot-air sterilizer, and it is advantageous when they have to be taken to a distant place to wrap them in paper which has been sterilized with them and which is only removed just before they are to be used. The paper, of course, is not necessary when the dishes, after cooling, are taken from the sterilizer and used at once in the laboratory.

FIG. 72



Petri dish.

Method of Pouring Plates or Preparing Petri Dishes.—This method must always be used when there is any chance of contamination of the material to be examined, and is at all times better than simply inoculating two tubes, as previously described. The method of preparing Petri dishes is practised as follows:

Take four agar tubes (if gelatin tubes are taken the procedure is the same, except as to the temperature figures given) and melt the contents in a water bath. Open one of the tubes and introduce a thermometer. This tube is not to be used for inoculation but merely for the control of the temperature. After the agar has been melted in all the tubes, allow it to cool down to about 50° C. Take the tubes out of the water bath. Flame the upper ends, remove the cotton plugs, and place the three tubes in a slanting position on the table. This can be done with the aid of an ordinary small slide box, paper box, or small wire rack. Inoculate tube No. 1 with platinum loop (properly sterilized) three times from the pus. Shake tube No. 1, always keeping it in a slanting position, so that microörganisms from the air cannot fall into it. Inoculate tube No. 2 three times with the platinum loop (previously sterilized) from tube No. 1. Shake the contents of No. 2 well and finally inoculate No. 3 from No. 2 in the same manner. Shake No. 3 well. Now pour the still fluid contents of the three tubes into three sterile Petri dishes ready for the purpose. This pouring of the Petri dishes is done as follows: Heat the upper

margin of the culture tube over a flame. Lift up the lid of the Petri dish a little, but be careful that it protects the dish from the microorganisms falling into it from the air. As soon as the fluid is transferred from the culture tube cover the Petri dish again with its lid. Do this with all three tubes and allow the agar in the dishes to cool as rapidly as possible. This is best accomplished by cooling them in the refrigerator before they are used. When the agar has set in the Petri dishes these can be labelled as Nos. 1, 2, and 3, corresponding to the tubes, and placed in the incubator, lids down, and the dish proper, containing the agar on top. This is done so that the condensed water which forms in the incubator will collect in the lid and not on the agar, where it would do harm.

Object of Preparing Petri Dishes.—This is explained by the following considerations: Tube No. 1 has been inoculated with pus from the abscess, and it would be reasonable to assume that several thousand cocci which are the cause of the suppurative abscess and a few hundred contaminating bacteria have been introduced. The pus has been diluted in the agar of tube No. 1, and when No. 2 has been inoculated from No. 1 there will be perhaps in No. 2 several hundred cocci and twenty to thirty of the contaminating organisms, while in tube No. 3, inoculated from the very dilute pus of No. 2, there will be only twenty cocci and one or two of the contaminating organisms. As soon as the agar has become solid, each individual bacterium, or perhaps a small group of individual bacteria which have clung together in spite of the energetic shaking and mixing, is fixed in a definite place, and at these places colonies of bacteria, composed of many millions, which can be seen either with the naked eye or with a hand lens or low magnification of the microscope, have developed from the single bacterium or small group within the next twenty-four to forty-eight hours. Petri dish No. 1, or, as it is often called, plate No. 1, will develop so many colonies in the incubator within the next twenty-four hours that they soon become confluent and cannot be picked up individually. Plate No. 3 is generally the best one. It contains perhaps ten to twenty colonies of the coccus and one or two colonies of the contaminating microörganism. The two different types can often be distinguished by the naked eye from the appearance of their colonies, but at times it may be necessary to make microscopic examinations before their character can be recognized. It must not be supposed that the organism present in twenty colonies is always the cause of the pathologic lesion, and that the one represented by one or two colonies is the contaminating microbe. Sometimes this is not the case, hence it is often necessary to find out by animal experiments which is the causative and which the contaminating bacterium. On the other hand, it is often possible to decide immediately which of the two is really the cause of the pathologic lesion. For instance, when examining the pus of an abscess as described above, and colonies of Staphylococcus pyogenes aureus and colonies of the hay bacillus are found, it

is obvious, of course, that the former pathogenic bacterium is responsible for the suppuration, while the latter harmless saprophyte represents an accidental contamination.

When there are on one or more of the Petri dishes *discrete* (*i. e.*, not confluent) colonies and the microscopic examination of stained cover-glass preparations shows these to contain one microörganism only which is considered the pathogenic causative bacterium and which is desired in pure culture, another set of Petri dishes should be prepared from this colony. This second set, provided it is inoculated from an uncontaminated colony, should contain colonies of one type only. From one of the colonies of the second set of plates a number of culture tubes of various media should then be inoculated for the further study and identification of the organism.

"Fishing" for Colonies.—It is sometimes not easy to find the young small colonies which may have developed after twenty-four hours in the Petri dishes, and it may not only be necessary to hunt for them with the low power of the microscope, but also to remove some of the material of such a colony with the platinum loop while looking through the instrument. This procedure is called *fishing* for the colony. It is not an easy one for the beginner, and in order to facilitate the work a special instrument with large stage and low-power lenses having a large field of vision is used.

Contamination with Moulds.—Plates and Petri dishes are often, in spite of all precautions, contaminated with moulds which have fallen from the air upon the culture soil. It is, as a rule, quite easy to distinguish the mould colonies from the bacterial colonies. The former, however, may grow very rapidly, and if moulds are discovered, subcultures, or transplants, should be made at once.

Modifications.—Sometimes it is very difficult to pour Petri dishes on account of the surroundings, as, for instance, in a barn. In such places a simple method of dilution is practised which often gives very good results. The method is as follows:

Have ready a number of slanting agar tubes. They may be kept upright in the vest or coat pocket if there is no chance to place them on a table. Sterilize the platinum loop, flame tube No. 1 over an alcohol lamp held by an assistant, open tube and inoculate the condensed water of the agar three times with the platinum loop. Sterilize the latter, open tube No 1 again, enter with a sterile loop and rub the condensed water well over the agar surface. Now inoculate tube No. 2 from No. 1. Sterilize loop again and enter tube No. 2 and rub over surface of agar and inoculate with the material obtained from the condensed water of tube No. 3. In this manner a dilution of the original material is obtained which will lead to the formation of a few discrete individual colonies in tube No. 3 or No. 4. If the tubes are dry and do not contain any condensed water, then the same method may be practised by simply rubbing the loop each time over the whole surface of the agar, sterilizing the platinum each time between

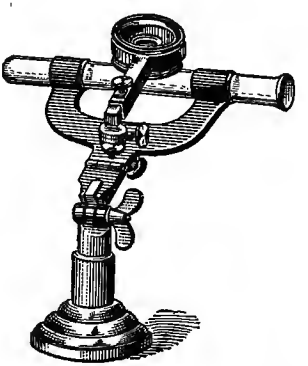
the inoculations of the different tubes. In this manner the desired dilution is obtained, and results in the formation of non-confluent individual colonies.

Another modification of the original plate method of Koch has been devised by Esmarch; its details are the following: Tubes with fluid, gelatin or agar, cooled down to near the point of solidification of the medium, are inoculated successively as described above, but their contents are not poured out. Instead the tubes are rolled on an ice block until the medium has formed a solid coating on the inside of the test-tube. The developing colonies can, as in the case of the plates or Petri dishes, be examined under the microscope.

FIG. 73

Method of holding tubes during inoculation. (McFarland.)

FIG. 74



Esmarch's magnifier for counting colonies of bacteria in Esmarch tubes. (McFarland.)

Impression Preparation.—When a plate or Petri dish has developed colonies it is often desirable to study the finer details of the arrangement of the bacteria forming the colony. This can be done by placing a clean dry cover-glass over the colony and pressing it down tolerably firmly on the culture soil. In this manner an impression of the colony on the cover-glass is obtained. The latter is then lifted with a pair of fine forceps, air dried, fixed, stained in the usual manner, and examined with the oil-immersion lens. The microscopic specimen obtained in this manner is known as an impression preparation or "Klatsch-Præparat."

Preparations from the Circulating Blood.—It is often desirable or necessary to make pure cultures from bacteria which may be present in the circulating blood of a sick animal or person. This is done in the following manner: The animal is first restrained. Next shave and cleanse the skin so that it is sterile. Have on hand a sterile (preferably all glass) syringe and a number of flasks containing from

50 to 100 c.c. of a fluid culture medium. When everything is ready, the syringe is taken out of the small instrument sterilizer or out of its receptacle in which it has been sterilized. The point of the needle is rapidly flamed and the needle is plunged into the animal's vein, which has been previously compressed centrally just beyond the point where the puncture is to be made. When the barrel of the syringe is full of blood and the needle has been withdrawn, the culture flasks are opened by an assistant and the blood transferred to them in the proportion of 1 to 5 c.c. to 50 to 100 c.c. of the fluid culture medium. The flasks are closed immediately, the cotton plugs flamed and the receptacles placed in the incubator. After twenty-four hours some of the contents of the flasks are poured into centrifuge tubes (carefully, so as not to permit any contamination from the air). The tubes are next centrifuged and cover-glasses, which are stained and examined in the usual manner, are prepared from the sediment. If any organisms are found, culture tubes with solid media are inoculated from the fluid media. It is also advisable to pour plates (Petri dishes) to detect contaminations if any are present.

Inoculation from Postmortem Material.—It often becomes necessary to obtain pure cultures from postmortem material. If the animal has not been dead long, or if immediately after death the body has been placed on ice, the procedure is not at all difficult, and is as follows: If the animal is a small one, not larger than a medium-sized dog, it is best to place the cadaver on a board and stretch out the four legs by fastening them with twine to four nails or blocks driven into the four corners of the board. An incision from the sternum to the symphysis pubis is made with a sterile knife and the skin is loosened from the thoracic and abdominal walls and tacked to the board. The peritoneum is then incised and the bony thoracic wall is removed by cutting through the costal cartilages. This is all done with sterile instruments which have been changed several times. When the heart is exposed it is raised with a pair of sterile forceps and the wall of the right ventricle is cut with sterile scissors or knife. As soon as the blood flows out, culture tubes are inoculated with it, using the platinum loop. Cultures may also be inoculated from the other organs with the aid of a very strong platinum loop. This is heated, and while still hot is pushed into the parenchyma of the organ, where it is left a few seconds until cool and then pushed in a little farther and small bits of tissue are withdrawn from the organ. With the particles so obtained culture media are inoculated. The procedure differs somewhat with large animals. The heart's blood is best obtained in the same manner, but the other organs should be removed and placed upon a table. A large flat knife is then heated over a flame and is pressed, while quite hot, on the organ. This singes the surface. The spot so treated is next cut into with a sterile knife and a platinum loop introduced into the incision to obtain some juice for inoculating culture tubes or pouring plates.

Subcultures, or Transplants.—After bacteria have been obtained in pure cultures by one of the methods described, it is always necessary to prepare, from time to time, fresh cultures, because numerous microörganisms soon show a tendency to die out in an artificial culture medium, on account of the accumulation of their own metabolic products or for other reasons. The transfer of an older culture to a new, fresh, sterile culture medium is made in such a manner that the danger of air and other contaminations is reduced to a minimum. This is accomplished by flaming the cotton and upper ends of the tubes or flasks containing the growth, as well as those of the new ones to be inoculated; or, when Petri dishes are used, by only lifting the lid sufficiently to allow the introduction of the platinum loop. The procedure of inoculating new tubes or flasks from preëxisting pure cultures is called making a subculture, or transplant (transplanting the cultures).

The first pure culture obtained from pathologic or other material is spoken of as the first generation; the first transplant as the second, the next transplant as the third generation, and so forth. A culture tube should always be so labelled that it clearly shows the kind of culture medium, the kind of microörganism, its derivation, the generation, the date when the original inoculation, or transplant, was made, and something about the manner in which it has been raised. A sufficiently designated culture would, for example, show the following inscription on the label:

Glycerin Agar
Bacillus Anthracis
Blood of mouse dead from Anthrax
Fourth Generation
January 15, 1910
(Incubator, kept aërobically)

It is, of course, not necessary to label as elaborately the cultures used by students for laboratory exercises, but the culture medium, the species of bacterium, and the date of inoculation must always be given, and they should be repeated on the label of the microscopic preparation. It is also desirable to indicate on the latter the stain used.

Streak Cultures.—The described method of making an original culture or a transplant by drawing the platinum loop over the slanting surface of agar or over a potato half-cylinder in a test-tube is called a streak culture. There is no special name for the inoculation of the fluid media.

Stab Cultures.—Gelatin of any kind and sugar agar are generally not prepared in test-tubes in a slanting position, but as a solid cylinder at the bottom of the tube. This condition is brought about by keeping the tubes in an upright position at the time when the media solidify. Such media are inoculated with the platinum wire straightened out into a needle. The outer free end of the wire, after sterilization over a flame and subsequent cooling, is brought in contact with the

bacterial growth and the needle is then plunged down vertically into the solid culture medium. This method of inoculating a tube is called a stick or stab culture. It is used when a gelatin tube is inoculated in order to show whether liquefaction takes place or not, and it is also employed in the preparation of anaërobic cultures, because the deeply inoculated bacteria are to a great extent removed from the air in the upper part of the tube.

Shake Cultures.—It is sometimes desirable to distribute the inoculated material evenly in a solid culture medium. The latter is then liquefied and cooled down to near its point of solidification. The inoculation is then made and the still fluid medium, after the closure of the tube with the cotton plug, is shaken violently and finally allowed to solidify in an upright position. Such a shake culture will readily permit the formation of gas bubbles in the developing growth.

Pure Cultures by Preliminary Animal Inoculations.—It is sometimes impossible to obtain the causative pathogenic bacteria in pure culture from a pathologic discharge, excretion, or tissue by direct inoculation of culture media. Even the plate (Petri dish) method is not available in some cases. The cause of tuberculosis, the tubercle bacillus, for instance, grows very slowly, and in tubercular discharge is generally associated with other bacteria. If tubes were inoculated and plates poured from a material of this kind all other bacteria present would develop days before the tubercle bacillus had a chance to form a colony. In a case of this kind it is necessary to inoculate the material into an animal susceptible to tuberculosis. The tubercle bacillus will multiply in its body and be present in certain locations and structures in pure culture. At an appropriate time the animal is killed and under aseptic precautions some of the tubercular material is obtained and brought into the proper culture media to give the tubercle bacillus a chance to develop and form a pure culture. The same method is generally necessary to obtain a pure culture of glanders bacilli from a horse suffering from glanders, because here also the glanderous discharges are contaminated by many bacteria growing more rapidly than the bacillus mallei. Further details about bacteria which have to be obtained in pure cultures in this indirect manner will be given in the chapters devoted to such particular microörganisms.

Anaërobic Cultures.—Anaërobic bacteria which do not develop in the presence of the oxygen of the atmospheric air have to be cultivated under special arrangements which will exclude this gas. Various methods have been developed to accomplish this end. The simplest method, devised by R. Koch, consists in placing a sterile piece of mica over the surface of a plate inoculated with an anaërobic germ. This method was later modified by replacing the mica by a sterile glass plate and sealing the latter around its margins by sterile gelatin or agar to which a small amount of some antiseptic had been added. Methods of this type have been largely abandoned, and have been replaced by better devices.

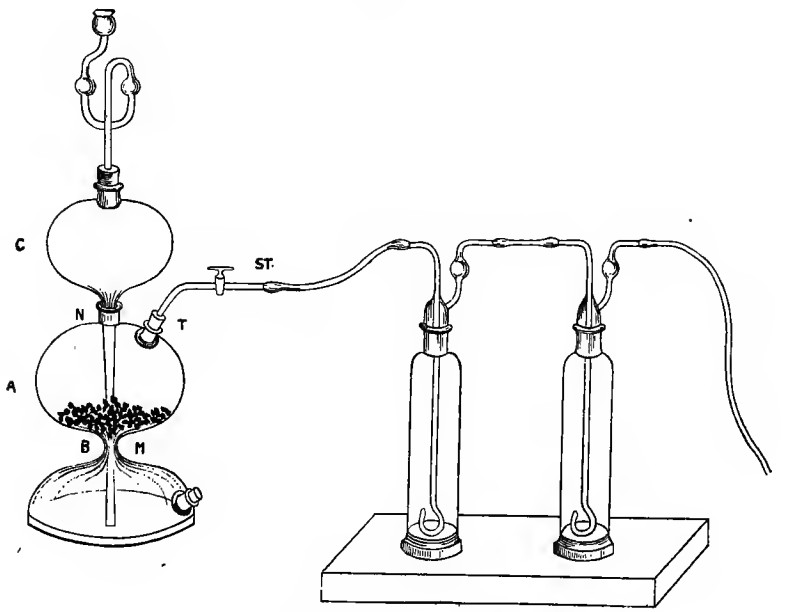
The Stick Culture Method.—Anaërobic cultures may be raised in stick cultures. For this purpose it is well to have a somewhat longer tube containing instead of 10 c.c., 15 to 20 c.c. of the culture medium. To the latter, when used to raise anaërobic stick cultures, a reducing substance, such as sugar, or, still better, formate of sodium, is often added. Anaërobic stick cultures can also be made as follows: A culture tube containing the usual amount of medium is inoculated as a stick culture. The medium in a second tube is melted and allowed to cool to near its point of solidification. Before this is reached, however, the cotton plug and the upper end of the tube are flamed, the former removed and the still fluid contents of the tube poured into the stick culture in the first tube. The added medium will successfully exclude the air from the lower inoculated strata, giving the anaërobic germ a chance to grow.

Exclusion of Air from Fluid Media.—A simple and often successful method of raising anaërobic germs in fluid media (bouillon, milk, etc.) is the following: The media, shortly before use, are subjected to a prolonged boiling, which drives out the atmospheric air. The culture flasks without being in any way disturbed or agitated, which would again mix the culture medium with atmospheric air, are allowed to cool, and are then at once inoculated. In the meantime there should be prepared some oil, vaselin or paraffin of a low melting point, which has been sterilized by heat and allowed to cool. As soon as the culture flasks have been inoculated some of the oil, vaselin or the like is poured into them. These substances, lighter than water, will float on the surface and exclude the culture medium and the anaërobic bacteria contained therein from contact with the atmospheric air.

Replacing the Air by a Hydrogen Atmosphere.—Anaërobic bacteria can be raised both in an atmosphere of hydrogen and in one composed of nitrogen from which all oxygen has been removed. When the ordinary air is to be replaced by hydrogen it is necessary to use an apparatus developing a continuous current of this gas. The simplest and safest device of this kind is a Kipp gas generator. It consists of two glass globes (*A* and *B*) joined together by a narrow neck and resting on a base. The upper globe (*A*) possesses a lateral tubular outlet (*T*) closed with a perforated rubber stopper which is provided with a glass tube and a stopcock (*ST*). A third globe (*C*) is generally shaped like a separatory funnel with a narrow conical glass tube fitting air-tight into the neck (*N*) of the upper globe (*A*) without completely closing the passage between the two jointed globes at (*M*). When this apparatus is to be used for the generation of hydrogen, the rubber stopper with the gascock at (*T*) is removed and pieces of broken glass are introduced in such a manner that they collect around the long glass tube in *A*. Next granulated zinc is introduced into globe *A* in the same manner. The zinc, provided that the broken glass has been arranged properly, cannot fall into *B*. The next step is to open the stop-cock at *ST* and fill *B* through the upper globular

funnel with enough sulphuric acid, considerably diluted (1 part H_2SO_4 to $9H_2O$)[1] until the fluid about reaches the constriction between the two united glass globes (at *M*). The stopcock is then closed and more dilute sulphuric acid is poured into the funnel (*C*), where it remains as long as the cock is closed. The tube with the stopcock is now connected with several Woulfe's wash bottles. This is done to remove impurities from the hydrogen gas. When the latter is to be generated the stopcock is opened, allowing dilute sulphuric acid to run from the upper funnel into the globe which contains the zinc. As soon as this takes place the development of hydrogen begins

FIG. 75



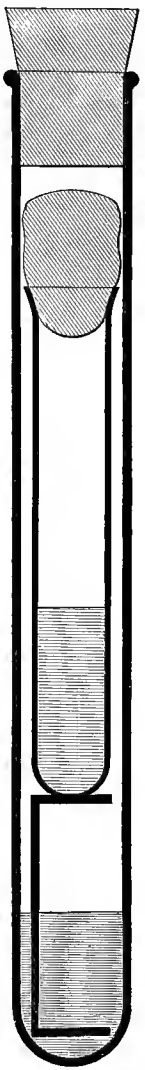
Kipp apparatus and accessories for generating and purifying hydrogen gas.

and the gas escapes at the open stopcock into the wash bottles, and from there into the tubes or jars which contain the media inoculated with anaërobic cultures. The flow of the gas can be regulated at the stopcock, which may be kept wide open or partially closed so that only a moderate amount of gas escapes. When no more gas is needed, all that is necessary is to close the stopcock at *st.* No more gas can escape, hence the hydrogen accumulates in A, and presses upon the

[1] When mixing H_2O and H_2SO_4 the sulphuric acid has to be poured slowly into the water. It is not permissible to pour water into strong H_2SO_4, because the latter may become so hot that dangerous consequences may be brought about. The dilute sulphuric acid must first have become cool before it can be used in the Kipp apparatus.

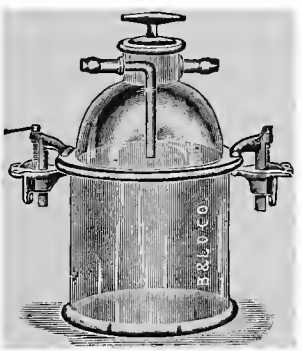
level of the fluid in *A* and displaces it downward. As soon as the fluid has receded from the zinc no more hydrogen is developed and an equilibrium of pressure is established in the apparatus. Whenever more hydrogen is needed all that is necessary is to open the stopcock, when the fluid from the funnel falls into the lower globe, rises into the upper globe, comes in contact with the zinc, and hydrogen is at once developed. When the zinc has all been dissolved, or when the dilute acid has become exhausted, it is necessary to refill the apparatus. When working with hydrogen it is necessary to remember that the gas is not only combustible, but forms, when mixed with the oxygen of the atmospheric air in the proportion of two volumes to one volume of oxygen, a very explosive gas. Hydrogen generated in the Kipp apparatus from ordinary commercial zinc contains, as contaminations, sulphur, arsenic, and also some oxygen. These bodies must all be removed before the gas can be used in the anaërobic cultures. This is accomplished by leading the impure oxygen through three wash bottles containing, respectively, the following solutions: No. 1, a 10 per cent. watery solution of nitrate of lead; No. 2, a 10 per cent. solution of nitrate of silver, and No. 3, an alkaline solution of pyrogallic acid. If chemically pure zinc and chemically pure sulphuric acid diluted with distilled water is used then

FIG. 77



Buchner's anaërobic tube. The fluid consists of pyrogallic acid dissolved in 10 per cent. soda solution. By Wilson's method the tubes are charged with pieces of caustic potash covered with pyrogallic acid. (Park.)

FIG. 76



Novy jar for anaërobic cultures.

the purification of the hydrogen is much simpler. The first wash bottle may then contain a solution of iodine and iodide of potash and the second one concentrated H_2SO_4. These bottles will wash and then dry the gas.

In order to replace the atmospheric air in the tubes, flasks, or Petri dishes containing the anaërobic cultures by hydrogen, special arrangements are always necessary to lead the gas in and then to close the culture medium container in an air-tight manner. The Novy jar is the apparatus easiest to handle. It comes in a high pattern adapted for tubes and flasks and in a low pattern adapted for Petri dishes. This jar has an inlet and an outlet tube which can be closed by air-tight glass stopcocks. After a Novy jar, containing culture tubes or plates, has been filled with hydrogen, it is well to seal the lid and the stopcocks with melted paraffin as an additional precaution. In order to see whether all atmospheric air has been displaced from the Novy jar or other apparatus used the following test should be made from time to time. The outlet tube of the jar is connected with a small rubber tube which carries at its outer end a small glass tube bent at right angles. The free limb of this rectangular glass tube is lead into a test-tube held with its closed end upward. After a short time the glass tube is withdrawn; the mouth of the test-tube rapidly closed with the thumb and the tube now inverted so that its mouth points upward. A match is lit and held at the mouth of the test-tube, and if the gas is pure it burns with a blue, non-luminous flame; if still mixed with atmospheric air there will be a slight explosion. This test, which should be repeated until the result indicates pure oxygen, must be made at some distance from the Kipp apparatus. The latter is best rigged up under a hood. Where none is present the escaping hydrogen can be let out of the room by the following simple arrangement: A small hole is made with an auger in a window frame and a glass tube passed through the hole. This glass tube is connected with the outlet tube of the Novy jar by a piece of small caliber rubber tubing and the hydrogen gas flowing through the apparatus is carried out of doors.

Removing the Oxygen from the Air by Chemical Means.—This method, first used by Buchner for the cultivation of anaërobic bacteria, is based upon the principle of absorbing the oxygen of the air in a closed vessel by an alkaline solution of pyrogallic acid. Applied to single-culture tubes the method is practised as follows: A large test-tube, into which the much smaller culture tube fits easily, is provided with a tightly fitting rubber stopper. One gram of pyrogallic acid and 10 c.c. of a 10 per cent. solution of potassium hydrate are placed in the large tube. The inoculated culture tube, with a piece of thin string fastened around the mouth, is suspended in the large tube and the string is held in place by the rubber stopper of the large tube. The latter is then sealed by pouring paraffin on top of the rubber stopper and around it. After this the tube can be incubated. If a

number of tubes or Petri dishes inoculated with anaërobic germs are to be treated by the pyrogallic-acid method, anatomical jars may be used for the pupose. Tubes can be placed in a slanting position against the wall of the jar, but when Petri dishes are used some device for them to rest upon should be placed in the bottom. The chemicals are placed in the jar, its lid screwed down and sealed with paraffin, and the jar is then ready to go into the incubator. A more elaborate glass jar is one made with shelves on which the plates containing the cultures may rest. This can be used with either the hydrogen or the pyrogallic-acid absorption method.

Anaërobic Cultures in Hen's Eggs.—Anaërobic cultures may also be raised in hen's eggs. The latter should be fresh and the shell must be cleansed externally by washing in a bichlorid solution and subsequently in sterile water, and finally drying with sterile cotton. A suitable spot is then perforated with a sterile needle and the inoculation is made with a slender platinum needle. The small hole is then closed with hot sealing wax and the whole outer surface coated with a varnish. Eggs so prepared are then incubated and at the proper time broken and their contents discharged into a sterile glass receptacle for microscopic examination.

The preparation of anaërobic cultures by the removal of the air from the container of the culture medium by an air pump is not often practised nowadays, since other anaërobic methods are much simpler and more preferable.

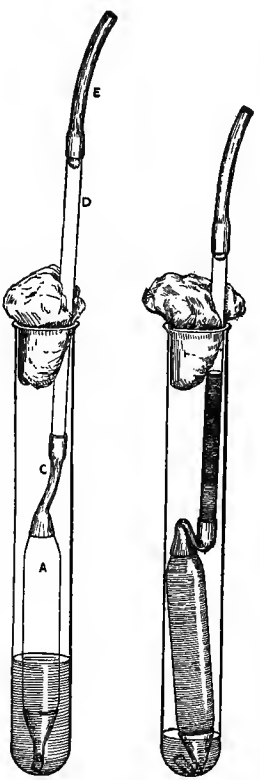
Wright's Method for Anaërobic Cultures.—Wright has devised two methods of developing anaërobic cultures: one of them is a modification of the pyrogallic-acid method of Buchner, the other consists in removing all air from the fluid culture medium by a special arrangement of the glass tube which contains the medium. These methods are described in Mallory and Wright's *Manual of Pathological Technique*. The first method is applicable to cultures in test-tubes and flasks; the details are as follows:

After the culture medium in the test-tube has been inoculated the cotton stopper is pushed into the test-tube, so that the top is about 1.5 cm. below the mouth of the test-tube. It is usually desirable to cut off a part of the protruding portion of the cotton before doing this. This cotton of the stopper should be of a kind that will readily absorb fluids. Now fill the space in the tube above the cotton stopper with dry pyrogallic acid and quickly add enough of a strong watery solution of sodium hydrate to dissolve it all. Avoid pouring on an excess; for a test-tube $\frac{3}{4}$ inch in diameter about 2 c.c. will be ample. Then, as quickly as possible, insert a rubber stopper firmly in the mouth of the tube so as to close it tightly. The culture is then ready to be set aside for development. The solution of sodium hydrate used consists of one part of the former dissolved in two parts of water. If done properly there is no danger of contaminating the culture medium from the alkaline pyrogallic-acid solution. The

method gives good results in obtaining pure cultures of the tetanus bacillus.

The other method of Wright's is as follows: The apparatus consists of a simple arrangement of glass and rubber tubes enclosed in an ordinary test-tube with a plug or cotton inserted in its mouth, as in an ordinary culture tube. The construction of the apparatus is shown in Fig. 78. *A* is a glass tube, somewhat constricted at each extremity. *B* and *C* are short pieces of small rubber tubing. *D* is a glass tube, in the upper extremity of which a small plug of cotton is inserted; *E* is a piece of rubber tubing. The test-tube contains a quantity of the fluid culture medium. When it is desired to make an anaërobic culture the fluid in the test-tube is inoculated in the usual way. The fluid is then sucked up into the system of glass and rubber tubes to a level above the rubber tube *C*. When it has reached this level the rubber tube *E* is compressed between the fingers to prevent the down flow of the fluid, and the system of tubes is then pushed downward in such a way as to bend the rubber tubes *B* and *C* as shown in Fig. 78. If the test-tube and the inner-tube system are of suitable size the rubber tubes mentioned will remain in this bent position. The fluid in the tube *A* is thus contained in a water-tight space, because the rubber tubes *B* and *C*, when bent to the angle shown in Fig. 78, are closed water-tight. Cover-glass preparations may be made from the culture fluid by straightening out the system of tubes and allowing the fluid in them to flow into the test-tube, where it is accessible to the platinum loop in the usual way. In using this method it is, of course, necessary that most of the air in the culture fluid be expelled before it is inoculated. This is easily done by boiling the culture fluid over the flame of a Bunsen burner without removing the inner system of tubes, and then cooling the apparatus by placing it in cold water.

FIG. 78



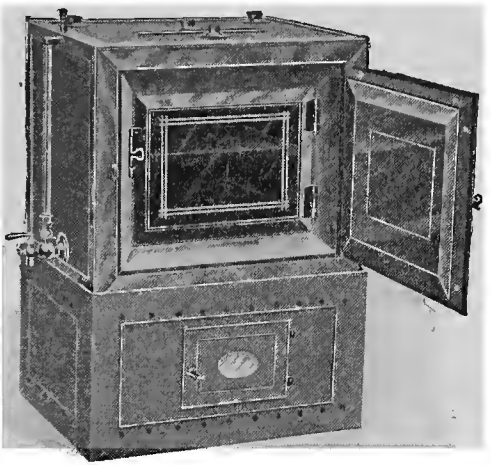
Wright's method of making anaërobic cultures in fluid media. (Mallory and Wright.)

Incubators or Thermostats.—Most pathogenic bacteria grow best at the temperature of the body of susceptible animals, and it is, therefore, necessary to raise artificial cultures in the incubator, thermostat, or brood oven at a temperature of about 37° to 38° C. Modern incubators for bacteriologic work are generally constructed of copper,

with double walls and double doors, and the space between the two walls is filled with water, a poor conductor of heat, which tends to keep a fairly steady, uniform temperature in the apparatus. The source of heat for a bacterial incubator is now, as a rule, illuminating gas, though some have an arrangement which will permit the use of a coal-oil lamp in places where gas is not accessible. The most modern incubators use electrical appliances as a source of heat.

Thermoregulator.—In order to keep a fairly uniform temperature in a bacterial incubator it should be so located that it is protected against very sudden changes of temperature and must be provided with a thermoregulator. The latter is a device or apparatus which will automatically regulate the supply of heat. Since incubators

FIG. 79



Thermostat, or incubator.

generally receive their source of heat from illuminating gas, most thermoregulators are designed to control the gas supply, which goes to the gas flame burning underneath the thermostat. The temperature in the incubator is controlled by a thermometer projecting through the upper double wall into the air space or chamber where the cultures are kept. The flame generally used under a thermostat is a so-called micro-gas lamp, a small burner which furnishes a small, narrow but high flame protected by a mica cylinder. Frequently a Koch-Pfeil safety lamp is used. This is so constructed that it will automatically shut off the gas supply if the flame should be blown out by a draft of air or for some other reason. However, if the thermostat is in a protected place this danger is very remote. A greater danger comes from leaks in the connecting rubber hose, and this must,

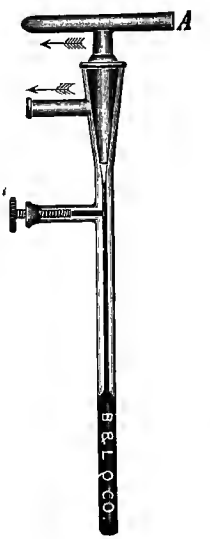
therefore, from time to time, be inspected. The sense of smell and a lighted candle applied carefully along the tube will detect any leakage.

The thermoregulators used in connection with thermostats with gas as a source of heat are generally constructed and based on the following principle: The gas is led into the upper part of the thermoregulator through a good dense hose, connected with an upper tube of the instrument, which is ground into the lower part. The vertical limb of the T-shaped upper part of the thermoregulator is drawn

FIG. 80

Incubator thermometer.

FIG. 81



Thermoregulator.

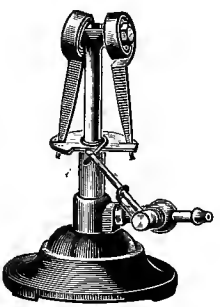
out conically and has an open tip and a number of small pinhole side openings. The lower, longer tube into which the T-tube fits is filled with mercury and the height of the column of mercury can be regulated by a screw working in a lateral branch of the lower sealed glass tube or mercury receptacle. If the column of mercury closes the lower opening of the T-tube then gas can escape into the lower tube only through the pinhole openings of the T-tube. The lower glass tube or receptacle for the mercury has, well above the level of the liquid, a lateral tube which is connected with the micro-gas lamp by a rubber hose. If the mercury shuts up the lower end of the T-tube very little gas can get to the lamp, and it burns with a small flame. If the mercury recedes from the lower end of the T-tube, gas can readily enter the lower glass tube and the gas lamp receives an abundant supply and burns with a large flame, giving much heat.

Starting of the Incubator.—The incubator is started in the following manner:

1. Immerse the thermoregulator up to the lower lateral branch, which carries the regulating screw in an almost completely filled flask. With the thermoregulator immerse a thermometer. Heat the water in the flask to 40° C., over a small flame which does not burn directly under either the thermometer or thermoregulator. Now regulate the screw on the lower lateral branch so that the mercury just closes the lower opening of the T-tube.

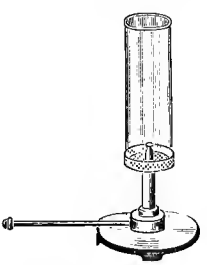
2. Pour water heated to about 40° C. into the compartment between the double walls of the incubator. This is done through an opening on top of the incubator, and it can be facilitated by using a funnel. When the compartment is completely filled, remove the funnel and close the opening with a cork having a small hole in the centre. Provide a perforated cork or rubber stopper for the thermoregulator and place it in a second opening near the margin of the incubator. The thermoregulator now dips into the water between the walls of the incubator.

Fig. 82



Koch's safety burner.

Fig. 83



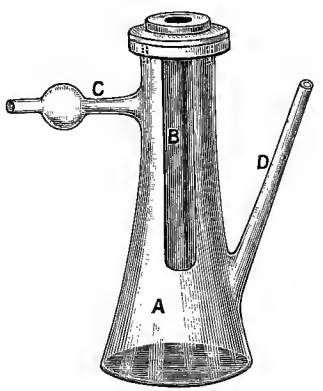
Micro-bunsen burner.

3. Connect the T-tube with the gas pipe and the median lateral tube with the micro-gas lamp by a rubber hose. Open the stopcock of the gas pipe and light the micro-gas lamp under the incubator. The latter will now burn with a small flame, because the thermoregulator dips into water about 40° C. and the mercury shuts up the larger opening of the T-tube. As soon as the water in the incubator is cooled off, the mercury recedes and the lower opening of the T-tube being now free, more gas goes to the lamp. If the thermoregulator has been set to 40° C., the temperature in the chamber of the incubator, indicated by the thermometer which projects into it, is generally about 37° C.

Difficulties in Regulating Incubators.—The student must not suppose that it is a very easy matter to regulate a thermostat so that it maintains a fairly stationary temperature. Among other things it is necessary to regulate the stopcock of the gas pipe so that the gas pressure in the thermoregulator is not too high. This pressure in most places, as well known, varies very much, and where this is

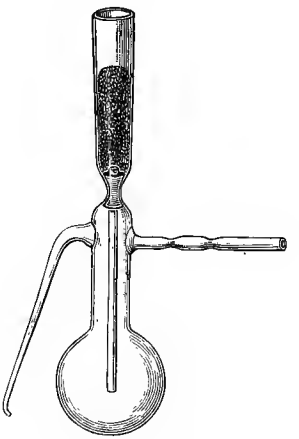
the case it is practically impossible to keep an incubator stationary. When a stationary temperature is of the utmost importance, as, for instance, in the preparation of attenuated cultures to be used as vaccines (anthrax vaccine), the gas cannot be used as it comes from the pipe, but must first be led into a gas-pressure regulator and from there to the thermoregulator. Electric thermoregulators for use in connection with gas lamps have also been constructed. The author has not yet had much experience with the electrical thermostat, where electricity furnishes the source of heat, and he does not know whether these are more reliable than the incubators heated by gas. Incubators are, of course, not all regulated to a temperature of 37° C.; they are frequently set for 22° C., and for special purposes at other temperatures.

FIG. 84



Reichel bacteriologic filter: *a*, receptacle; *b*, porcelain filter; *c*, arm for vacuum pump; *d*, outlet.

FIG. 85

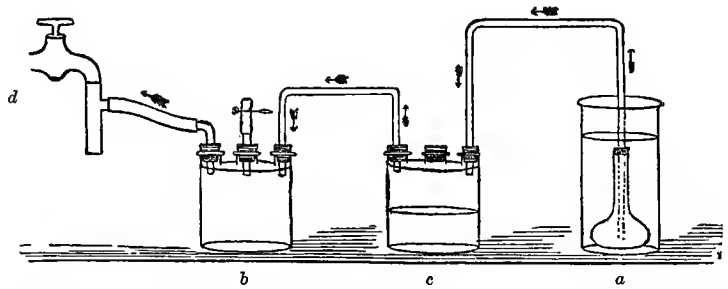


Pasteur culture filter.

Bacteria Filters.—In order to separate soluble toxins from the bacteria which have produced them in fluid culture media, it is necessary to filter the latter. Even the best and densest filter papers would not do for this purpose, since bacteria can pass through the pores. Pasteur, Chamberland, Berkefeld, and others have devised filter masses which are so dense that bacteria cannot pass through them. Kaolin, clay, and other similar substances, moulded into the form of bougies, cylinders, or flasks, are the masses used. They are not glazed, of course, because this would make them impervious to a watery fluid. Such filters vary in the size of their pores, and those having the smallest will not permit even the most minute microorganisms to pass. The Pasteur-Chamberland filter generally has smaller pores than the Berkefeld filter. Whenever a fluid medium in which bacteria have been grown is filtered for the purpose of obtain-

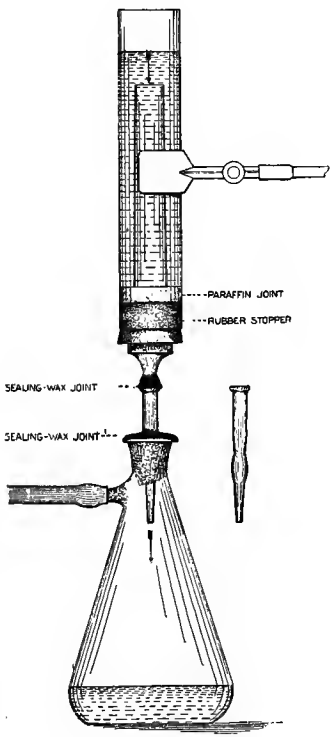
ing a bacteria-free toxic liquid or with some other object in view the filtrate must always be tested to find out whether any bacteria are present. This is done by inoculating from the filtrate solid culture

FIG. 86



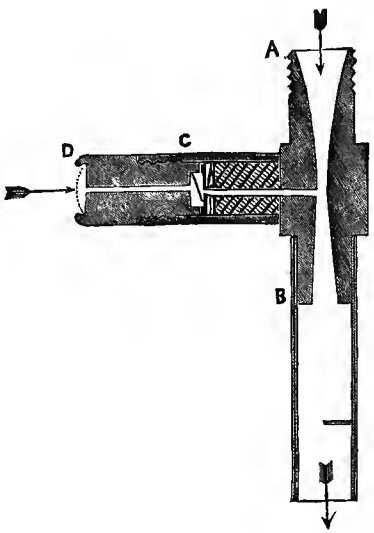
Apparatus for the rapid filtration of toxins, etc.: *a*, filter flask; *b*, Woulfe bottle to guard against regurgitation of water from the pump; c, reservoir for the filtrate; *d*, water vacuum pump. (McFarland.)

FIG. 87



Chamberland filter.

FIG. 88



Brass suction pump: *a*, connection to faucet; *d*, connection for rubber tube from filtering apparatus; *c*, valve to prevent regurgitation of water to filter.

media, and by placing part of it in the incubator to see whether it will become cloudy and whether anything will develop in it.

Such filtrates are commonly said to be germ-free or sterile. The former term is correct provided it refers to visible germs of known type only. The term sterile, however, is objectionable, because it is known today that there are ultramicroscopic invisible filterable organisms which are evidently able to multiply and cause diseases, such as hog cholera, rinderpest, pleuropneumonia of cattle, etc. Since at present it is not known whether bacteria-free filtrates may not contain other ultramicroscopic live substances which so far cannot be demonstrated and recognized, the term sterile should not be used in connection with filtrates.

The fluids which are to pass through Pasteur or other filters must be subjected to pressure because gravity alone will not force them through rapidly enough. Therefore, such bacteria filters are generally connected with a suction pump screwed to a faucet, or they are attached to a vacuum apparatus which exhausts the air by a steam or gas engine or electrical device. In any case a partial vacuum is formed in the vessel which is to receive the filtrate, and the external air pressure acting upon the fluid to be filtered, presses it through the pores of the filtering device. It is, of course, understood that all parts of a filtering apparatus must be connected with each other in an absolutely air-tight manner; otherwise, filtration is not perfect and the filtrate may become contaminated with bacteria from aspirated air.

QUESTIONS.

1. Describe the method of obtaining a pure culture of a bacterium suspected of being the cause of an abscess which has not yet broken through or ulcerated.
2. Describe the same process in the case of an ulcerated abscess.
3. What is meant by pouring plates? What has superseded the original Koch method of pouring plates?
4. Describe a Petri dish. How are these prepared for use?
5. What is meant by flaming the upper end of a culture tube? When practised?
6. Describe methods of preparing culture tubes in order to pour plates from their contents.
7. Describe procedure of inoculating a set of three tubes from which plates are to be poured.
8. How are the Petri dishes treated after the liquefied agar medium has been poured into them?
9. What is the object of pouring plates (Petri dishes)?
10. What is the difference in development of colonies in Petri dish No. 1, No. 2, and No. 3, respectively?
11. How can it be ascertained when several different types of colonies are present; which is the causative pathogenic and which are the accidental contaminating microörganisms?
12. How are young small colonies found in a Petri dish?
13. What kind of contamination is frequently found in Petri dishes?
14. If the environment makes the use of Petri dishes impossible, how can a pure culture be obtained?
15. What is Esmarch's method of obtaining pure cultures?
16. What is an impression or "Klatsch" preparation?

17. Describe method of obtaining a pure culture from bacteria present in the circulating blood of an animal.
18. How is a microscopic preparation made from fluid culture media when only a few microörganisms are thought to be present?
19. Describe a bacteriologic postmortem examination with the preparation of cultures from the heart's blood and from various internal organs.
20. What is meant by a subculture? What other term for subculture is in common use?
21. Why is it necessary to make subcultures frequently?
22. What is meant by the first, the third, the twentieth generation of a pathogenic bacterium?
23. How should a culture be labeled? How a microscopic stained preparation?
24. What is a streak culture? What is a stab culture?
25. How is a "shake" culture prepared and for what purpose?
26. How would you prepare a pure culture from a discharge containing glanders or tubercle bacilli? Give reasons for the procedure adopted.
27. What is an anaërobic culture?
28. What are the principles of the methods used in preparing anaërobic cultures?
29. What was Koch's first method to exclude atmospheric air from a plate culture?
30. How is the stick culture method practised for the preparation of anaërobic cultures?
31. What is the simplest method of raising anaërobic bacteria in fluid culture media?
32. Describe the method of using a Kipp gas generator for the development of hydrogen gas.
33. What precautions are necessary in mixing water and sulphuric acid and why?
34. What is a Novy jar. Describe its use in connection with a Kipp apparatus.
35. What chemical means are used for removing the oxygen from the atmospheric air in a closed vessel?
36. How are these chemical means made use of for a single culture tube and how for a number of them and for Petri dishes?
37. How is an anaërobic culture prepared in a hen's egg?
38. Describe Wright's method of preparing anaërobic cultures in completely filled glass tubes.
39. Describe Wright's modification of the Buchner method of raising anaërobic cultures in tubes.
40. What other names are in use for a bacterial brood oven?
41. What is the construction of such apparatus?
42. What is a micro-gas lamp?
43. What is a thermoregulator? Describe the glass mercury thermoregulator.
44. How is it adjusted for use with an incubator kept at 37° C?
45. Describe the method to start an incubator to be kept at 37° C.
46. How are the incubator gas hose and glass tubes inspected for leaks?
47. Under what circumstances is it particularly important to keep the thermostat always at a uniform temperature?
48. Describe method used to obtain germ-free filtrates of bacterial cultures.

CHAPTER XIII.

IDENTIFICATION OF BACTERIA—CULTURAL CHARACTERISTICS—ANIMAL EXPERIMENTS.

In practical bacteriological work it is not merely sufficient to obtain a bacterium in pure culture from a pathologic product or other source, but the organism must also be fully identified. This may be a very easy matter, requiring perhaps only a simple animal experiment or a simple serum test. On the other hand it may require the study of the pure culture on a variety of media, and under varying conditions, with careful observation of the naked-eye appearances of the growth, tests for metabolic products and microscopic examination in the hanging drop and the examination of specimens stained by different methods. Certain cultural peculiarities, like the liquefaction of gelatin and the production of acids and gases, have already been considered. It is also necessary to note the effect of certain growing bacteria on the fluid culture media. For example, the media may appear clear, with the formation of a sediment or cloudy, either slight or heavy. It should also be observed, when a sediment is present, whether it is granular or not, whether a pellicle is formed, and whether, in the latter case, streaks from it reach to the bottom of the tube or flask; also, whether and when the pellicle sinks to the bottom. The precipitation of the casein in milk and the tendency of certain growths on a solid medium to adhere to the platinum loop when introduced must also be noticed. Any and all of these features may be quite characteristic of certain bacteria, and may give considerable aid in identification. The colonies on plates and slants present certain characteristics which must be observed in reflected and transmitted light. Their size, color, dryness or moisture pigments and early and late tendency to confluence are often important characteristics. A number of descriptive terms applied to colonies and bacterial growth as a whole in or on various culture media must now be explained.

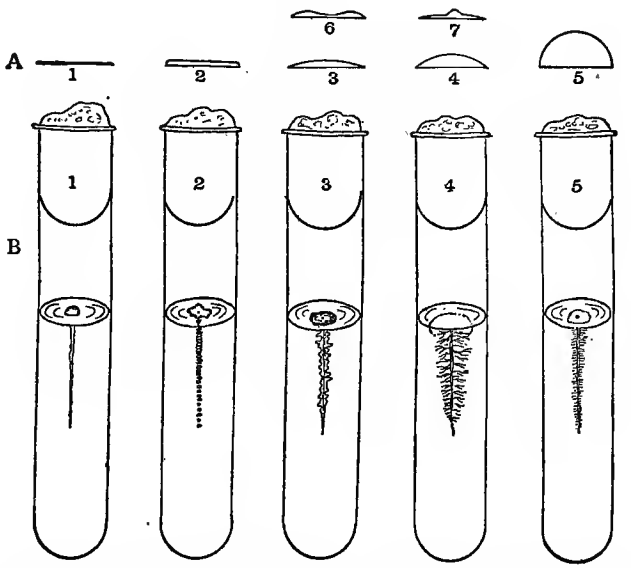
CULTURAL CHARACTERISTICS.

Stab Cultures.—Stab cultures in gelatin which do not liquefy the medium, and which show a uniform growth along the stab, without any special characters, are known as *filiform* growths. The growth is called *nodose* when it is composed of closely aggregated colonies,

and *beaded* when the loosely placed colonies can be distinguished individually. When the colonies are arranged in such a manner that there is some fancied resemblance to papillary excrescences they are known as *papillate.* An *echinate* growth indicates one beset with sharp extensions which radiate from the centre into the culture medium. A *villous* growth shows some resemblance in its arrangement to the villi of the intestines. *Arborescent* means branched like a tree, and *plumose* denotes a delicate feathery growth.

When liquefaction occurs in the gelatin the liquefied zone is likely to show very definite arrangements and shapes, to which the following descriptive terms are applied:

FIG. 89



Showing characters of gelatin stab cultures: *A*, characters of surface elevation: 1, flat; 2, raised; 3, convex; 4, pulvinate; 5, capitate; 6, umbilicate; 7, umbonate. *B*, characters of growth in depth: 1, filiform; 2, beaded; 3, tuberculate-ecinulate; 4, arborescent; 5, villous. (From Chester.)

Crateriform, a flat excavation like a saucer or crater.

Tubular, cylindrical, saccate, elongated areas of liquefaction.

Infundibular or *conical,* more or less funnel-shaped areas of liquefaction.

Fusiform or *spindle-shaped,* those which have the greatest diameter in the middle and taper both upward and downward.

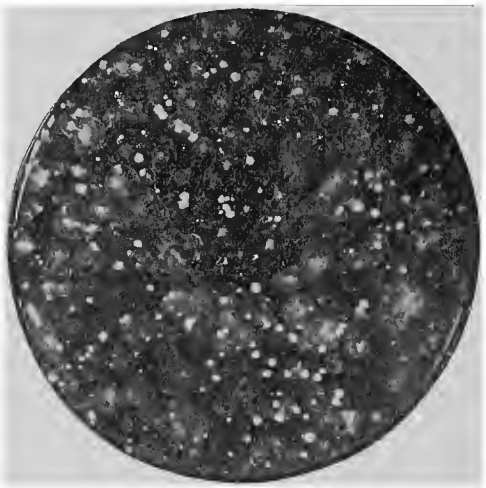
In *stratiform* liquefaction the whole mass at the upper end of the gelatin tube becomes fluid and the process progresses downward, involving deeper and deeper strata of the culture medium.

The liquefied gelatin may be comparatively clear, with a sediment

at the deepest portion, it may contain flocculi, it may be uniformly cloudy and turbid, and it may finally be covered by a pellicle.

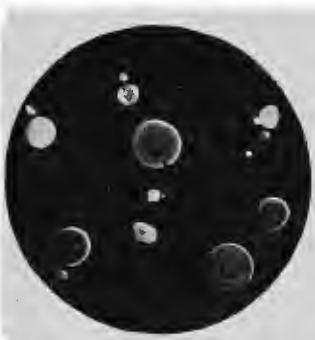
Shake Cultures.—In gelatin shake cultures, gas bubbles, liquefaction, or an abundant growth toward the surface are distinguished. The latter indicates an aërobic development, while an abundant growth in the lowest strata points to an anaërobic bacterium.

FIG. 90



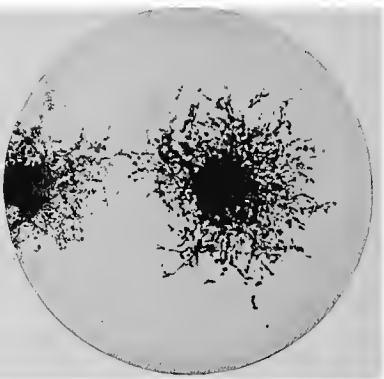
Photographs of a large number of colonies developing in a layer of gelatin contained in a Petri dish. Some colonies are only pinpoint in size; some are as large as a pencil. The colonies here appear in their actual size. (Park.)

FIG. 91



Well-distributed colonies on agar in Petri dish. (Park.)

FIG. 92



Irregular fringed colony (B. malignant edema). (From Kolle and Wassermann.)

Streak Cultures.—In streak cultures on slanting agar or blood-serum tubes, filiform, echinate, beaded, effuse, and arborescent growths are

distinguished. The condition of the condensed water in the tubes is also noted, and whether it is clear or cloudy, or whether there is a precipitate, and whether the latter is *granular, flocculent,* or *slimy,* or shows any other particular quality.

Plates.—On plates are studied the individual colonies in particular, their size is noted, the following features are distinguished, and the following descriptive terms are used:

Punctiform denotes the dimensions so small that they cannot be well measured by the naked eye.

Round, elliptical, fusiform, irregular are self-explanatory terms; *cochleate* is a colony which is twisted somewhat like the shell of a snail.

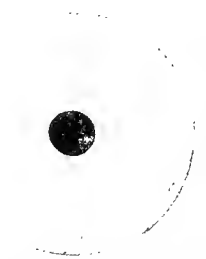
Ameboid is a colony irregular in outlines and with processes looking somewhat like the pseudopodia of an ameba.

Myceloid is a colony of bacteria with radiating slender masses looking more or less like a mould mycelium.

FIG. 93

Round surface colony, colon bacilli grown in stiff gelatin. (Park.)

FIG. 94



Colony of typhoid in rather stiff gelatin. (Park.)

FIG. 95

Colonies of typhoid and colon bacilli in rather soft gelatin. (Park.)

Filamentous colonies are composed of an irregular mass of loosely woven filaments.

Floccose indicates a dense woolly structure.

Rhizoid denotes an irregularly branched, root-like character.

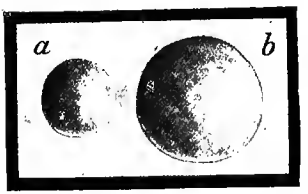
Conglomerate is an aggregation of small colonies more or less equal in size and character which present one compound larger colony.

Toruloid are colonies composed of several small round or oval colonies, arranged like a group of budding torula or yeast cells.

Rosulate means shaped like a rosette.

Sometimes terms indicating a fancied resemblance to certain pictorial objects are used; anthrax colonies, for example, are sometimes spoken of as being like the head of *Medusa,* because under low power they show tortuous twisted strings and masses of bacilli at the margin which somewhat resembles the mythologic serpent-surrounded head of a Medusa. With reference to their elevation over the surface, colonies are described as *flat, raised, convex, pulvinate, hemispheric,* or *capitate;* also *umbilicated,* having an umbilicus-like impression, and *umbonate,* having a central nipple-like elevation or knob.

FIG. 96



Moist raised colonies with no visible structure, looking like a drop of water.

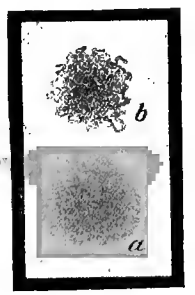
FIG. 97

Deep colonies, usually either light brown, gray, or yellow in color, opaque, with little marking.

FIG. 98

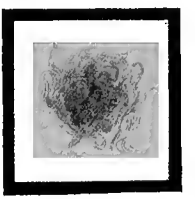
The colonies very finely granular, with or without twisted threads at borders.

FIG. 99



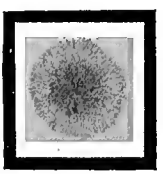
Colony in gelatin. The centre is coarsely granular in partly fluid gelatin. The borders are formed of wavy bands of threads.

FIG. 100



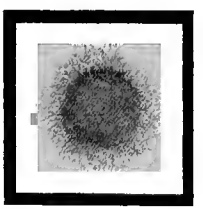
Colonies opaque in centre, with lighter borders. The margin is coarsely granular.

FIG. 101



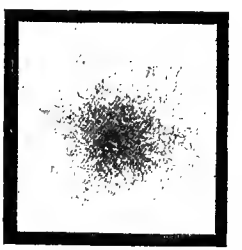
Colonies circular in form, composed of radiating threads.

FIG. 102



Colonies with opaque centres, with a thin border fringe.

FIG. 103



Colony showing a network of threads which is thicker in centre.

Figs. 96 to 103 from Lehman and Neumann.

Surface Details and Peripheral Outlines.—In addition to the term *smooth*, the following surface details and peripheral outlines are distinguished.

Alveolate, marked by depressions so that a somewhat honeycombed appearance results.

Punctate, dotted with punctures like small pinholes.

Bullate, irregular elevations somewhat resembling a blistered surface.

Vesicular, looking like small vesicles, and due to gas formation.

Verrucose, wart-like, with papillary prominences.

Squamous or *scaly*, covered with scales.

Echinate, beset with pointed prominences.

Papillate, beset with nipple-like prominences.

Rugose, presenting short, irregular folds, in consequence of the shrinkage of the surface growth.

Corrugated, arranged in long folds.

Edges.—The edges of the colonies are described as *entire* when there are no divisions and no serrations; as *undulate* when the outlines are wavy; as *repand* when they are like the border of an open umbrella; as *ciliate* when they show hair-like extensions.

Details.—The finer internal details of the colonies are studied on microscopic impression preparations, and the following descriptive terms are used:

A *reticulate* structure shows the form of a network like the veins of a leaf.

Areolate, divided into rather irregular or angular spaces by more or less definite boundaries.

Gyrose, marked by wavy irregular lines like the convolutions of the brain.

Marmorated, marked like marble.

ANIMAL EXPERIMENTS.

Animal experiments are frequently made in order to obtain bacteria in pure culture, to identify pathogenic bacteria beyond doubt, to test methods of disinfection or sterilization, to control the attenuation of vaccines, to obtain immune sera, etc. To obtain a pure culture of glanders from an open lesion due to the Bacillus mallei, it is generally necessary to inoculate a male guinea-pig in the particular manner described in the chapter on glanders. The inoculation of a guinea-pig or a mouse makes the rapid diagnosis of a doubtful case of anthrax possible; similarly, guinea-pigs must be inoculated in order to ascertain whether pasteurization has killed tubercle bacilli in milk. After the preparation of anthrax vaccines it is necessary to estimate accurately the attenuation of the bacilli by injecting them into mice, guinea-pigs, and rabbits. When testing

tetanus and diphtheria antitoxins, varying proportions of antitoxin-toxin mixtures must be injected into guinea-pigs. It will thus be seen that numerous circumstances arise in practical bacteriology when the animal experiment is absolutely necessary and unavoidable. Animal inoculations are also necessary in the preparation of antitoxins, of antirabic virus, in the immunization against Texas fever, rinderpest, etc.

In conducting experiments on animals it is generally necessary first to restrain them. Numerous operating tables, both large and small, with restraining devices and holders for mice and guinea-pigs and larger animals, have been constructed. Generally elaborate devices are unnecessary, and a few boards of varying sizes with four nails or blocks driven into the corners will suffice. To the latter the outstretched legs of the animal are fastened with twine. The principal instruments used in inoculation experiments are hypodermic syringes; however, operating knives, scissors, forceps, needles, suturing material, and even trephines may be needed in certain procedures. The most important factor in all animal experiments is the necessity of strictest asepsis, in order that accidental and misleading results may not be obtained. Injection of a bacterial culture, pathogenic excretion, blood, milk, etc., into an animal requires shaving and thorough antiseptic cleansing of the part to be inoculated. The inoculation is made with a sterile syringe, and the operator must work with clean, antiseptically treated hands.

Subcutaneous Inoculation.—Subcutaneous inoculation is one of the most common methods practised. In its simplest form it consists of a slight incision in the skin without any aseptic precautions, followed by rubbing a little of the suspected material into the wound. This method is sufficient for the experimental identification of anthrax or bubonic plague bacilli. In other instances a *deep pocket* must be made by pushing a probe under the skin through a small incision and lifting up the skin, forming thus a deep protected pocket. This method is used for inoculating material suspected of containing tetanus bacilli or spores.

When the subcutaneous method is used with asepsis the skin must be shaved and cleansed and a sterile hypodermic needle employed. In using hypodermic syringes the operator must be careful not to contaminate himself with the dangerous bacteria which he may be handling. A good syringe with a tightly closing piston should be used. Immediately after the injection the entire instrument, including the needle, should be placed in a vessel with water that is slightly alkaline and boiled. The operator must then thoroughly cleanse his hands in a strong bichloride solution.

Intravenous Inoculation.—Another method of inoculation frequently used is the intravenous injection in which the material is injected directly into a vein. In most animals the jugular can generally be used for this purpose. The skin over it is shaved and cleansed anti-

septically and compression is made central to the point of injection. In large animals it may be advantageous, first, to expose the vein by an incision in the skin and fascia. If this is necessary, it must, of course, be done with sterile instruments. Rabbits are generally inoculated intravenously through the large veins of the ear. The posterior vein is better adapted for injections than the larger anterior. The injection is made from the external surface of the ear, where the hair should be clipped in order to facilitate sterilization and also the finding and proper compression of the vein. It is best to use a small, short injection needle. Blood may also be withdrawn by this same method. This is necessary in cases where the immunizing serum of a treated rabbit is tested before finally killing it and collecting all the serum.

Intraperitoneal Inoculation.—This is very frequently practised in bacteriologic tests. In addition to the usual asepsis, it is important to make the injection in such a manner as not to injure the intestines. This may be accomplished by either one of two methods. One is by the use of dull needles, which require a preliminary incision through the skin and fascia in the median line of the anterior abdominal wall. The needle is then pushed right in the median line through the peritoneum and the overlying tissues. In the other method the animal is placed hind legs upward, a position in which the intestines fall toward the diaphragm. If the needle is introduced in the middle line below the umbilicus, and held very obliquely so that it does not penetrate very far into the abdominal cavity, the danger of injuring the intestines or any of the abdominal viscera is reduced to a minimum.

Other Forms of Inoculation.—Inoculations are occasionally made in the thoracic cavity through an *intercostal space,* but more frequently in the *anterior chamber of the eye,* where the developing lesions can be studied directly from day to day. *Subdural* inoculations are made after preliminary incision in the scalp and trephining of the skull. Injections into the *spinal canal* may be made in the lumbar region, by inserting a long needle of the hypodermic syringe into the canal, between two vertebræ, laterally from the median line.

Infections of the Intestinal tract are made by feeding animals with the infected material. If it is desirable to introduce such material directly into the intestines, it is necessary to perform a *laparotomy,* exposing the duodenum, into which the injection is made directly with a very small hypodermic syringe.

Animals are sometimes infected experimentally through the *respiratory tract.* This is done by connecting their cages, directly or indirectly, with a spraying apparatus which disseminates the infected material.

Quantity of Culture Inoculated.—It is generally desirable, except in purely diagnostic work, to use a definite quantity of the pure culture for inoculation. This may be accomplished by a variety of methods: (1) A pure culture is prepared by inoculating 10 c.c. of bouillon.

This is kept in the incubator for twenty-four hours, then well shaken, and a definite fraction of the whole amount used for each animal. (2) An agar tube with a slanting surface is inoculated by rubbing the material thoroughly over the entire surface with the platinum loop. After incubating for twenty-four hours the whole growth is removed with the platinum loop and intimately and uniformly mixed with 10 c.c. of an 0.85 per cent. salt solution, a fraction of which is finally inoculated into the experimental animals. (3) *A platinum loop of definitely known size* is used for removing a portion of the growth from an agar slant. One holding just 2 mg. of a bacterial growth is known as a "*Normaloese*," or "*Normal loop.*" To make these loops a little apparatus consisting of a number of steel rods held in small wooden blocks has been constructed. By winding the free end of the platinum wire around the smallest steel rod a loop is formed which will just hold 2 mg.; the other steel rods form loops holding, respectively, 2, 5, and 10 mg. The quantity of bacterial growth removed from an agar slant with such a "*Normaloese*" is well rubbed up with 2 c.c. of physiologic salt solution and the entire mixture is injected. An animal so treated is said to have received one, two, or five, as the case may be, "Normaloesen" of a certain bacterial growth.

Collodion Sacs.—It is sometimes desirable to implant bacteria into the body of an animal in such a manner that they are protected against the phagocytes of that animal. This is done by the aid of collodion sacs whose walls permit osmotic processes to continue but prevent the emigration of bacteria and the entrance of leukocytes and other cells. The simplest method for preparing them is as follows: Clean a small test-tube and dry it completely by washing first in absolute alcohol and then in ether. Pour some fairly thick collodion, or celloidin as used in section work, into the dry tube and move it continually in such a manner that the collodion coats its entire interior. When the collodion becomes very thick in consequence of evaporation, let the last few drops run over the rim at the mouth to the outside of the tube. The tube is then filled with water, and after a little while the outside collodion is peeled off without tearing it from its connection with the inside collodion. The collodion surrounding the inner mouth of the tube is loosened, and by allowing water from the faucet to flow between it and the test-tube wall it gradually separates. A slight pulling on the collodion is often necessary completely to detach it from the tube. A glass tube slightly smaller than the test-tube is now prepared, and near one end a narrow constriction is blown in over a flame. About $1\frac{1}{2}$ inches of the lower end of the collodion sac is now cut off and the open end is slipped over the constricted glass tube and fastened to it with a piece of good surgical silk. Finally, fresh thick collodion is painted over the silk and the upper rim of the sac over the glass tube, making a water-tight connection between the sac and glass tube. Of course, each sac must be tested before it is slipped over the glass tube. A number of them should be prepared, as only

a portion will be of such quality that they can be used. The collodion sacs are then filled with nutrient bouillon and placed in larger test tubes containing the same bouillon. Both the glass tube and the test tube are cotton plugged, and then sterilized and cooled. Later the collodion sacs can be inoculated with the platinum rod. They must then be taken out of the tubes and dried externally with sterile cotton and the constriction heated over a flame, drawn out and securely sealed. They are now ready for implantation into the peritoneal cavity of an animal.

Non-pathogenic bacteria in collodion sacs implanted in the peritoneal cavity of an animal can be so changed that they acquire pathogenic properties, and after removal from the animal and subsequent inoculation in another animal of the same species they will produce disease because they are able to multiply and resist phagocytosis.

QUESTIONS.

1. How can a bacterium obtained in pure culture from some pathologic product be fully identified?
2. What are the different changes in appearances produced by bacteria (*a*) in nutrient bouillon? (*b*) in milk?
3. Explain the meaning of the following terms used with reference to a non-liquefying growth of a bacterium in gelatin: Filiform, nodose, beaded, papillate, echinate, villous, arborescent, plumose.
4. Explain the meaning of the following terms, used with reference to the liquefying growths in gelatin: Crateriform, tubular, cylindrical, saccate, infundibular, conical, fusiform, stratiform liquefaction.
5. What features should be noted in a gelatin shake culture?
6. What features should be noted in the condensed water of an agar slant?
7. What arrangement offers the best chances to study the characteristics of individual colonies?
8. Explain the following terms used in the description of bacterial colonies: Round, elliptical, fusiform, irregular, cochleate, ameboid, myceloid, filamentous, floccose, rhizoid, conglomerate, toruloid, rosulate.
9. What is meant by a colony "resembling the head of a Medusa?" Name a bacterium forming such colonies.
10. What terms are used with reference to the different types of elevations of colonies over the surface of the culture medium?
11. What are the meanings of the following terms: Alveolate, punctate, bullate, vesicular, verrucose, squamous, echinate, papillate, rugose, corrugate?
12. What terms are used in the description of the margins of colonies?
13. Explain the following terms: Reticulate, areolate, gyrose, marmorated.
14. For what purposes are animal experiments made in bacteriologic studies.
15. Describe the subcutaneous method of inoculation.
16. The intraperitoneal method.
17. The intravenous method.
18. The subdural method. The method of inoculating into the spinal canal.
19. How is a small animal restrained for inoculation?
20. What precautions are used to avoid injuries in intraperitoneal inoculation?
21. What is a "Normaloese?" What is the object of using it?
22. What other methods are used to inoculate a definite amount of a bacterial growth?
23. Describe the method of preparing collodion sacs.
24. What is the object of using them?

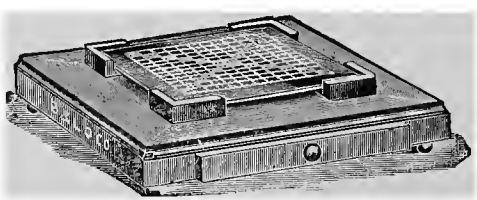
CHAPTER XIV.

METHODS OF EXAMINING AIR, SOIL, WATER AND OTHER FLUIDS FOR BACTERIA.

The general principle underlying the examination of air, soil, water, and other fluids is that of mixing a definite amount of these substances with a suitable culture medium, pouring plates or Petri dishes, and studying the developing colonies of bacteria, yeast cells, moulds, etc., as to species and numbers of colonies developed. In the case of substances like soil, water, milk, and other fluids a small definite amount can be taken directly and mixed with the culture medium. In the case of the air, a known volume must be aspirated either into a culture medium or into a sterile bland substance which is subsequently mixed with a culture medium.

The methods of examination, however, vary a good deal according to the particular object in view. If a soil is to be examined for the presence of tetanus, anthrax or malignant œdema bacilli, some of the material is inoculated directly into susceptible animals (mice, guinea-pigs, etc.) without first resorting to cultural methods. Likewise, in the examination of air for tubercle bacilli (first extensively carried on by Cornet) the dust which is aspirated with the air or which has settled spontaneously from it must be secured and inoculated directly into guinea-pigs.

Fig. 104



Wolffhügel's apparatus for counting colonies.

Counting the Colonies.—In the examination of air, soil, water, milk, etc., for microörganisms (bacteria, yeast cells, moulds, etc.) it is often desirable to obtain as exact a numerical estimate as possible. This is accomplished after proceeding as indicated above by counting the number of colonies developed in the Petri dishes.

Wolffhügel's Counting Apparatus.—Colonies are generally counted with the aid of a Wolffhügel counting apparatus, which consists of a

black glass plate, contained in a wooden frame. The Petri dish is placed on the black glass. Above it, resting on four blocks, is a transparent glass plate which has been ruled with a diamond into square centimeters and fractions of a square centimeter. The colonies in one cubic square, both on the surface and in the depth of the medium are counted with the aid of a hand magnifying glass or the low-power lens of a compound microscope with a large stage. This is done for a number of squares and the average number of colonies per square centimeter is then calculated. If it has not been done previously, the exact diameter of the lower part of the Petri dish is then measured and the surface of the culture medium contained in the lower dish is calculated according to the formula for the surface of a circle, which is $r^2 \pi$. For example: Counting 5 square centimeters:

Square No. 1	16 colonies
Square No. 2	23 colonies
Square No. 3	14 colonies
Square No. 4	18 colonies
Square No. 5	19 colonies
Total	90 colonies
Average per square cm.	18 colonies
Diameter of lower part of Petri dish	6 cm.
Hence radius	3 cm.
Hence surface 9×3.14	28.26 cm.
This figure multiplied by 18 equals	308.68 colonies

This means that the Petri dish has developed 309 colonies; and if 1 c.c. of water was mixed with the culture medium, the result shows that this water did contain 309 live bacteria per cubic centimeter.

Esmarch's Apparatus.—Sometimes, in the examination of air, water, etc., plates cannot be poured. In such cases Esmarch role-tubes or other glass tubes coated on the inside with the culture medium must be prepared for the development of the colonies. With tubes the Wolffhügel counting apparatus is replaced by a special magnifying glass with tube holder, devised by Esmarch. The outside of the glass tube is divided into a number of equal fields. The colonies in a number of fields are counted, the average is obtained and multiplied by the total number of fields. In other words, the fields are treated the same as the squares in the preceding example.

These methods furnish an approximate result only, but one that is accurate enough for the purpose.

Bacteriologic Examination of Air.—When a very exact quantitative bacteriologic examination of air is unnecessary the method practised by Robert Koch will be found both simple and satisfactory. Culture media are poured into Petri dishes and allowed to solidify. The lid of the dish is then removed and the culture medium exposed to the air for a definite period of time (for instance, ten or fifteen minutes). The lid is then replaced and the Petri dish is kept at room temperature or incubated in the usual manner. By this simple method compara-

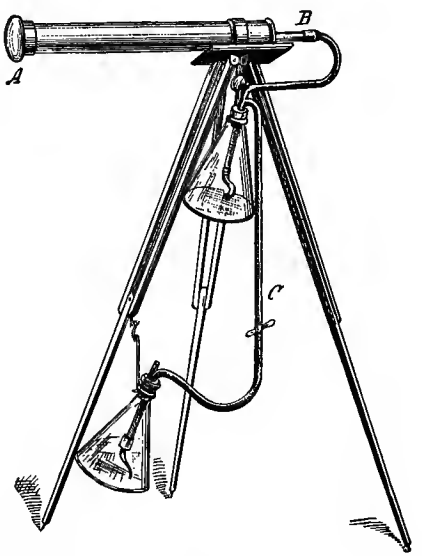
tive studies of the air can be made in the open, in a room, in a barn, in a basement, etc., as the difference in the number of colonies developed in Petri dishes exposed simultaneously, or approximately so, to air under different conditions for the same period of time, gives a fairly accurate indication of the variations in the bacterial contents of the air. Data as to the relation between the number of bacteria and moulds present can also be obtained by this method.

EXACT METHOD.—For the exact quantitative estimation of bacteria and moulds a variety of methods have been devised. The simplest of these, which is, however, only slightly more accurate than the one first described, is as follows. A large Erlenmeyer flask, preferably one holding at least 2 liters (2000 c.c.), is filled with water, cotton plugged, and thoroughly sterilized in the steam sterilizer. After being removed from the latter the water is cooled and the flask taken to the place where the air is to be examined. A test tube containing 25 to 30 c.c. of agar or gelatin is heated in a water bath and cooled down to near the point of solidification. When this is ready the cotton plug is removed from the flask and the sterile water poured out, which is now, of course, replaced by two liters of air. The flask must be energetically shaken with the mouth down in order to get it as dry as possible. It is then placed on a level surface. The melted culture medium poured in, the cotton plug replaced, and the medium allowed to solidify. The flask must be kept perfectly quiet, either at room or incubator temperature, which causes the microörganisms contained in the air to fall to the bottom and develop into colonies on the culture medium. Before using the latter for this purpose it should have been kept in the incubator for several days, so that all of the water of condensation has evaporated and the medium is comparatively dry. Otherwise, the water of condensation is squeezed out of the solidifying medium and running over its surface will interfere with the formation of the proper number of colonies. After remaining in the incubator for a few days the flask is taken out and the colonies which have developed in the medium on the bottom can be counted with a magnifying glass. More exact methods for counting the number of bacteria and moulds in the air are the following:

HESSE'S METHOD.—The apparatus consists of a glass tube 70 cm. long and 3.5 cm. wide, the interior of which is coated with a thin layer of gelatin. One end of the tube is closed by a solid rubber stopper or cap, the other by a perforated rubber cork which carries a small glass tube. This latter is connected by a rubber hose with a suction bottle or flask which is in turn connected with a second flask of the same type, each of exactly 2 liters' capacity. After the long glass tube has been properly sterilized in the hot-air sterilizer and coated with gelatin it is mounted in a horizontal position and one of the suction flasks is connected with the small glass tube in the perforated rubber stopper. This flask is filled with water and connected with the second suction flask, which is empty, in the manner

shown in Fig. 105. The rubber cap is then removed from the end of the horizontal tube and enough suction by mouth made at the exit tube of the lower and empty flask to start the water running from the upper flask. The water then siphons out of the higher flask into the lower one, and during this process two liters of air are aspirated through the gelatin coated tube. The outer end of the tube is then closed with the rubber cap and the suction flasks reversed. The cap is again removed, and as suction is made on the empty flask the water flows as before from the upper to the lower flask and two more liters of air are aspirated through the gelatin-coated tube. The operation is repeated a number of times until about 20 liters of air have been

FIG. 105



Hesse's apparatus for collecting bacteria from the air. (McFarland.)

aspirated The long tube is then disconnected, closed at both ends with sterile cotton plugs, and kept in a horizontal position for several days to permit the colonies to develop. These are then counted. During the aspiration the water should flow at such a rate that 1 liter passes from the upper to the lower flask in one to two minutes.

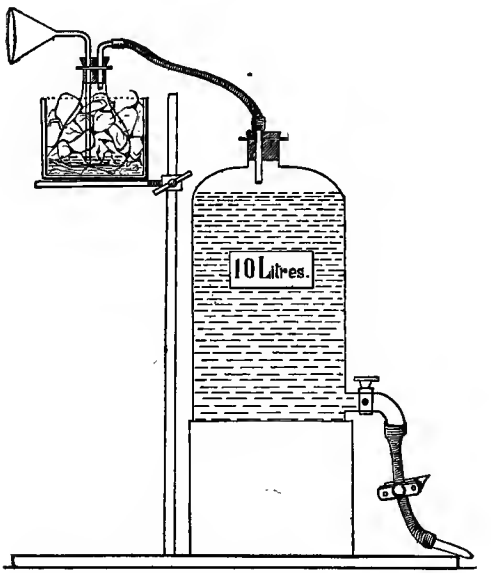
EYRE'S METHOD.—Eyre recommends the following apparatus and method of quantitative bacteriologic examination of air:

Apparatus.—1. Aspirator bottle, 10 liters' capacity, fitted with a delivery tube, and having its mouth closed with a perforated rubber stopper, through which a short length of glass tubing passes.

2. Erlenmeyer flask, 250 c.c. capacity (having a wide mouth, properly plugged with cotton), containing 50 c.c. sterile bouillon.

3. Rubber stopper to fit the mouth of the flask, perforated with two holes, and fitted as follows: Take a piece of glass tubing, 15 cm. in length and bend up 3 cm. at either end at right angles to the main length of tubing. Pass one of the bent ends through one of the perforations in the stopper; plug the opposite end with cotton. Take a glass funnel, 5 or 6 cm. in diameter, with a stem 15 cm. long, and bend the stem close up to the apex of the funnel in a gentle curve through a quarter of a circle. Pass the long stem through the other perforation of the rubber stopper. In addition, rubber tubing, screw clamps and spring clips for tubing, a steam sterilizer, retort stand and clamps are required.

FIG. 106



Arrangement of apparatus for air analysis. (Eyre.)

Method of Procedure.—1. Fill the aspirating bottle with 10 liters of water and attach a piece of rubber tubing with a screw clamp to the delivery tube. Regulate the screw clamp by actual experiment so that the tube delivers 1 c.c. of water per second. At this rate the aspirator bottle is emptied in just under three hours. Close the rubber tube below the clamp by means of a spring clip, and make up the contents of the aspirator bottle to 10 liters.

2. Sterilize the fitted rubber cork with its funnel and tubing by boiling in the steam sterilizer for ten minutes.

3. Remove the cotton plug from the 250 c.c. Erlenmeyer flask containing 50 c.c. sterile bouillon and replace it by the rubber stopper with its funnel and bent tube. Make sure that the end of the stem of the funnel is immersed in the bouillon.

4. Place the Erlenmeyer flask with the bouillon in a glass, metal, or other suitable vessel, and pack it around with cracked ice. Place the Erlenmeyer flask on a stand or box so that the bent glass tube in the perforated stopper can be conveniently connected by a rubber tube with the aspirating bottle.

5. Remove the spring clip from the rubber tube and allow the water to run from the aspirating bottle at the rate of 1 c.c. a second, as previously arranged.

6. From time to time replace the ice in order to keep the bouillon near 0° C. in order to prevent multiplication of the bacteria in it.

6. When the 10 liters of water have run out of the aspirating bottle, a corresponding quantity of air has been aspirated through the 50 c.c. of bouillon in the Erlenmeyer flask. The bouillon now contains all the bacteria that were originally in the 10 liters of aspirated air. Then disconnect the Erlenmeyer flask with the 50 c.c. of bouillon and shake the contents well.

7. In the meantime a number of gelatin or agar tubes have been liquefied and cooled down to near the point of solidification of the media. Then with sterile graduated pipette add some of the bouillon to the fluid culture media in these tubes. To the first add 0.5 c.c., to the next 0.3 c.c., and to a third 0.2 c.c. The contents of the tubes are next poured into two sets of sterile Petri dishes, one of which is kept at room and the other at incubator temperature. After a few days the colonies are counted and the average per cubic centimeter of bouillon is calculated. This average multiplied by fifty indicates the number of bacteria contained in the 50 c.c. of bouillon in the Erlenmeyer flask and represents the bacteria present in the 10 liters of air which have been aspirated through the apparatus. The number of bacteria present in air is usually stated in terms of cubic meters, and since a cubic meter is equal to 1000 liters, the last figure must be multiplied by 100 to obtain the number of bacteria in one cubic meter.

Methods of Frankland and Petri.—Frankland and Petri have, independent of each other, devised a method in which the air is first aspirated into a filter of sterile quartz sand. The grains of sand have an average diameter of $\frac{1}{3}$ to $\frac{1}{4}$ mm., and are contained in a piece of glass tubing 6 to 10 cm. long and about 2 cm. wide. The sand is held in the centre of the tube, and divided into two equal portions by fine wire gauze. One end of the tube is closed by a cotton plug and the other, after thorough dry sterilization, by a perforated rubber stopper containing a small glass tube. When the device is to be used the latter is connected with an air pump. The tube containing the sand filter is held upright during the process of aspiration which is continued at the rate of 10 liters per minute for from ten to twenty minutes. The upper and lower portions of sand are then separately mixed with liquefied gelatin or agar, well shaken, and poured into Petri dishes. The lower portion of sand should be found sterile, the

upper portion alone developing colonies. These are counted in the usual manner.

Modifications of the above method have been introduced by Ficker, who uses glass powder as the filter mass, and by Frankland, who uses sterile powdered sugar.

Sedgwick and Tucker have modified the filtering device by providing at the upper end an expanded portion ruled outside in equal squares. After aspiration of the air, gelatin is poured directly into the tube. As soon as the sugar powder loaded with the air bacteria is dissolved the tube is rolled on ice and becomes an Esmarch roll-tube. This method avoids the transfer of the filter material into a separate plate or tube.

Bacteriologic Examination of Water.—*Collection of Water.*—The collection of water differs according to the source from which it is obtained. Two factors must be particularly considered: (1) Care must be exercised that the water running into a sterile flask does not wash into it bacteria which may have been on the outside of the mouth of the vessel. The risk can easily be avoided by sterilizing the flasks in the dry air sterilizer and wrapping them in paper which is not removed until the actual moment of use. (2) Care must be taken that water coming from a faucet does not collect from the mouth of the latter bacteria which may have been deposited here by some means other than the water itself.

Operating and dressing rooms in hospitals are now frequently provided with hot and cold sterile water, which must be examined from time to time. In the bacteriologic examination of such water the author has used the following method in order to exclude the possibility of contamination from outside sources. A 150 c.c. beaker is filled with 95 per cent. carbolic acid and held so that the mouth of the faucet dips into the acid. After the latter has acted for several minutes the beaker is removed and replaced by one containing hot sterile water. This removes the carbolic acid. The faucet is next opened and the water allowed to flow for several minutes. A sterile flask is then partially filled and immediately closed with a cotton plug.

When water has to be collected from a river, a large water tank, or a pond, it is desirable to obtain the specimen at some distance from the shore or wall of the tank. This can be accomplished by fastening a string to a sterile flask containing lead shot, and throwing the vessel out into the water. When filled it is rapidly withdrawn, and after a little water has been poured out the flask is closed with a sterile cotton plug. For obtaining water from a particular depth of a body of water, special apparatuses have been constructed by Esmarch and by Roux. Esmarch's device consists of a bottle with a rubber cap and weight. It is lowered on a line to a definite depth, the rubber cap is opened by a string attached to it, the bottle is filled, the rubber cap closed again automatically, and the apparatus is pulled up to the surface.

Roux uses flasks which are drawn out at the neck into a thin twisted capillary glass tube. After sterilization they should contain a small amount of distilled water, which is heated over an open flame to boiling and evaporated down to a small residue. The capillary tube is then fused over a flame and the bottles cooled, when they will be found to contain a vacuum. A heavy string is attached to the closed capillary tube and the bottle enclosed in a metal capsule, which is lowered into the water. When the apparatus is at the desired depth the string is pulled, breaking the capillary tube and allowing the water to rush into the vacuum in the flask. The apparatus is then drawn to the surface.

A trustworthy bacteriologic examination of water can only be made if the plates are prepared on the spot where the samples are collected. If water is removed to a distance, even when packed in ice, the subsequent count of the colonies does not furnish an accurate result, because some water bacteria multiply near the freezing point, while others are killed by chilling.

Inoculation of Culture Media.—The culture media must be inoculated with definite amounts of the water. For this purpose 1 c.c. pipettes, subdivided into 0.1 and 0.01 c.c., are required. These pipettes should be placed in glass tubes which are fused at one end and closed with a cotton plug at the other. The upper ends of the pipettes themselves must also be closed with cotton plugs, and they and the tubes sterilized in the hot-air sterilizer. Water containing few bacteria may be mixed with the culture media in quantities of between 1.0 and 0.1 c.c. When there are many bacteria, as in the case with water contaminated by sewage, the sample must be diluted with sterile distilled water before being mixed with the culture media. The sterile water is brought to the place of examination in volumetric flasks in quantities of 25 c.c., 50 c.c., 100 c.c., and 250 c.c. One c.c. of the suspected water is added to the sterile distilled water in the volumetric flask. The latter is well shaken and 1 c.c. of the diluted sample is added to the culture medium with a fresh sterile pipette. The culture media used for the bacterial examination of water should be of a very definite degree of alkalinity. It is necessary to use both gelatin and agar plates. They should be kept at a temperature of 20° C. for eight days, since many water bacteria grow slowly on artificial culture media. If water is examined with special reference to certain pathogenic bacteria, special culture media and incubator temperature are necessary. The most important pathogenic bacteria occurring in water are the typhoid bacillus and the cholera spirillum.

Bacteriologic Examination of Milk.—The bacteriology of milk will be treated in subsequent chapters.

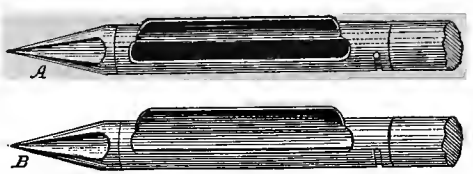
Bacteriologic Examination of Soil.—*Qualitative Examination.*—As already stated this is chiefly undertaken for the detection of pathogenic bacteria like those of anthrax, tetanus, and malignant edema.

These microörganisms are most readily detected by direct animal inoculations. The search for nitrifying and other bacteria, important in the study of agricultural problems, requires certain special culture media and the dilution of the finely divided soil with sterile distilled water.

Quantitative Examination.—This is very unreliable and generally not very satisfactory. In the first place it is difficult to divide the soil so finely that it can be intimately mixed with melted culture media, and, again, the soil bacteria (mostly bacilli) have such a wide range of conditions of growth that no single cultural method can furnish even an approximate picture of the species and number of germs present in a specimen of soil. For example, there are aërobic and anaërobic bacteria, some growing at very low and others at extremely high temperatures.

Specimens of soil from the superficial strata are generally obtained with a small platinum spoon or scoop containing, when filled, about $\frac{1}{50}$ or $\frac{1}{25}$ of a cubic centimeter. The material may first be rubbed up in a sterile agate mortar, or it may be mixed directly by violent shaking with the melted culture medium, which is subsequently poured into Petri dishes. For the examination of soil of deeper strata a drill or auger has been devised by Fränkel. Above the tip is a metal tube with an opening on one side, with a cover arrangement on hinges like a door. During the downward movement the door remains closed, but as soon as the motion is reversed it opens and the tube is filled with soil. In this manner it is possible to obtain soil from a definite depth.

FIG. 107



Tip of Fränkel's instrument for obtaining earth from various depths for bacteriologic study. *B* shows the instrument with its cavity closed, as it appears during boring: *A*, open, as it appears when twisted in the other direction to collect the earth. (McFarland.)

Bacteriologic examination has shown that soils are generally richer in bacteria the more they have been mixed or contaminated with manure, sewage, and other decomposing vegetable or animal material; further, that this richness is confined to the superficial layers. At a depth of 1 meter the number of bacteria is much reduced, and at a depth of $1\frac{1}{2}$ to 2 meters very few are present; still deeper there are none at all.

QUESTIONS.

1. What is the general principle underlying the bacteriologic examination of soil, water, milk?
2. What is the principle on which the method for quantitative bacteriologic air examination is based?
3. What is meant by a quantitative, by a qualitative bacteriologic examination of soil, water, air, etc.?
4. What method is used when soil is to be examined for the presence of tetanus bacilli?
5. How can air be examined for the presence of tubercle bacilli?
6. Describe the arrangement and use of a Wolffhügel counting apparatus.
7. What is a square centimeter; what a square millimeter? What is a cubic centimeter?
8. How many colonies are in the culture medium of a Petri dish 8 cm. in diameter if on an average each square centimeter contains twenty-three colonies?
9. What is an Esmarch roll-tube?
10. Describe a simple method of approximately estimating the number of germs in air by the aid of exposed Petri dishes.
11. Describe another method by the aid of large Erlenmeyer flasks filled with water.
12. What is a liter? What is its equivalent in pints?
13. Describe Hesse's method of quantitative bacteriologic air examination.
14. Describe Eyre's method of aspirating air for a quantitative bacteriologic analysis.
15. Describe the method of using sand or sugar as filters for catching the bacteria in aspirated air.
16. What is the Sedgwick-Tucker modification of the air-filtering tube?
17. How are flasks used in collecting water for quantitative bacterial analysis to be sterilized and handled before use?
18. How can water running from a faucet be prevented from washing down bacteria from the latter into the collecting flask?
19. How is water collected from a river or pond?
20. What devices are used for collecting water from a particular depth?
21. Describe the method of obtaining definite amounts of water from the collecting bottles for the inoculation of culture media.
22. What procedure is employed with a specimen of water containing many bacteria in order to avoid getting an uncountable number of colonies in the Petri dishes?
23. At what temperature and how long shall the Petri dishes be kept before the count of the colonies is undertaken?
24. Why do quantitative bacterial soil examinations furnish very unreliable figures?
25. Describe the method of collecting soil from the surface. Also method of obtaining soil from any desired depth.

CHAPTER XV.

PRINCIPLES OF DISINFECTION—DISINFECTANTS.

WHEREVER there is disease due to pathogenic microörganisms the latter will, through exhalation, secretions, and excretions, direct or indirect contact, transport by insects or otherwise, soil or contaminate objects in the neighborhood of the sick animal. When stables, barns, harness, feed, water supply, or any other objects are soiled or contaminated with pathogenic bacteria they are said to be *infected.* The removal or destruction of such infecting bacteria is called *disinfection.* Disinfection may sometimes take place without destruction of the bacteria, as in the filtration of water through filters so dense as to prevent the passage of the microörganisms. As a rule, however, the object of disinfection is to destroy the pathogenic bacteria. Physical and chemical means may be employed for this purpose. Among the former, heat and sunlight are particularly important; among the latter such chemicals as accomplish the object in comparatively weak concentrations.

Effectiveness of Disinfectants.—The effect of every disinfectant must be studied separately for each type of pathogenic organism and also for the vegetative forms and spores of sporulating bacteria. The spores are, as has been previously pointed out, much more resistant to all disinfecting procedures than the vegetative forms. In the theoretical laboratory study of disinfection it is necessary to ascertain not merely the circumstances under which physical or chemical means kill all bacteria, but also to find out at what degree or concentration they have a marked hindering or inhibiting influence upon bacterial growth and multiplication. In practice the absolute killing of all infecting bacteria is seldom possible. Diluting, diminishing, and damaging them in such a way that their growth is inhibited and they are robbed of much of their virulency must generally be considered as sufficient. The simplest and most potent physical agent used for disinfection is heat. Bacteria grow best at a certain temperature, called their optimum temperature; any degree above it retards their growth, and any degree above their maximum temperature inhibits growth entirely and generally damages them seriously. When working with chemical agents the least concentration which will entirely inhibit growth must first be ascertained, and then the concentration which will destroy bacteria and the necessary time to accomplish it. Absolutely harmless, non-pathogenic bacteria, some of which form spores more resistant than the spores of any pathogenic

bacteria are not considered in disinfection. The destruction of all life in a medium, as has already been explained, is called sterilization; disinfection, however, does not go to this extent.

The determination of the exact disinfecting value of either physical or chemical agents is more difficult than would appear on first sight. It is always necessary in such experiments to use bacteria which have been grown under the most favorable conditions and which are derived from a young, vigorous culture. Another important factor is whether these bacteria are contained in distilled water, salt solution, or a favorable fluid culture medium, because in the latter they are generally more resistant than in pure water. It is also necessary to protect them from any damaging influences before the actual test.

Theobald Smith has shown that tetanus spores obtained under the most favorable conditions can resist moist heat for over one hour, while those raised in media containing sugar and previously damaged by the acid which they have formed from it are much less resistant.

The action of the disinfectant is also much influenced by the presence of certain substances in the culture medium. Corrosive sublimate, for example, is much weakened in the presence of albuminoid or proteid bodies or discharges containing them. After the test the removal of the disinfectant from the bacteria by washing or chemical means is another important factor.

Testing the Effect of Temperature.—The method used for testing the effect of temperature upon bacteria is the following: Culture tubes containing a few cubic centimeters of young bouillon cultures of the organism are removed from the incubator and placed in a water bath containing a large amount of water heated to a certain stationary temperature. One of the tubes must contain a thermometer in order to indicate the exact moment when the temperature in the culture medium coincides with that of the water bath. The tubes are then heated for varying periods of time—for example, for five, seven, ten, eleven, and twelve minutes, and so on. They are taken out of the water bath one by one and at once plunged into cold water. Upon the conclusion of this process, culture tubes with liquefied solid media are inoculated, plates are poured, and the developing colonies are subsequently counted. The first plate remaining sterile indicates the time exposure necessary to kill the organism at the temperature used in the experiment. The author has found this method inaccurate by furnishing values which are too low, because the small quantity of the original cultures transferred in pouring the Petri dishes may not contain any live bacteria, though an appreciable number of especially resistant individual organisms may be present in the whole bulk of the bouillon. An absolutely trustworthy method is the following:

The tubes are heated and cooled as previously described, and then the entire contents of each tube (for example, 5 or 10 c.c.), after

flaming the mouth, is poured into a flask containing 100 c.c. of sterile bouillon. The flasks are incubated for forty-eight hours and solid media are inoculated from these. By this method even a single surviving bacterium is given an opportunity for subsequent development. The effect of sunlight and other physical influences may be ascertained in an identical manner.

Testing the Effect of Chemical Disinfectants or Antiseptics.—The method generally employed is the following: Sterile silk threads are soaked in culture media containing the bacteria whose resistance is to be tested. The threads are afterward dried and suspended for different periods of time in solutions of varying strength of the antiseptic. The latter is then washed off in sterile distilled water and the silk threads are immersed in fluid or liquefied solid culture media. Since there are certain objectionable features in the silk-thread method it has been modified, and small sterile garnet crystals on which the bacteria are dried are used to replace the threads. The crystals are treated in the same manner as the threads. All chemical disinfectants act more quickly at high than at low temperatures; that is at high temperatures which are not in themselves damaging. This observation corresponds with the general law that chemical reactions take place more promptly and more rapidly at higher temperatures.

Dry and Moist Heat.—One of the most important considerations in the study of heat in disinfection is the fact that dry heat is very much less effective than moist heat. This is particularly apparent in the case of spores. Anthrax spores, for instance, can withstand dry heat at 100° to 120° C. for several hours, and even at 140° C. their absolute destruction is only accomplished after three hours, while water boiling at 100° C. kills them, even at the highest estimate, in twelve minutes. The effect of steam developed under pressure in the autoclave is still more powerful. The spores of certain soil and potato bacilli can withstand streaming steam at 100° C. for over sixteen hours, but they are killed in steam under pressure at 105° to 110° C. in from five to fifteen minutes and at 140° C. in one minute. Steam developed under reduced pressure at a lower temperature, as at high altitudes or in experimental work is, of course, less effective in killing spores than steam at the ordinary pressure of one atmosphere and at 100° C. Overheated steam not saturating the atmosphere in which the spores are exposed also has a reduced destructive power. Esmarch and Kokubo found that a very small admixture of antiseptics, (creosote, formalin, etc.) to the streaming steam, enormously increased its destructive power toward spores. Dry heat is not only much less powerful as a disinfectant than moist heat, but is also relatively of much less value because it has little penetrating power. Koch and Wolffhügel showed experimentally that in a bale composed of nineteen woollen blankets, which had been exposed for three hours to dry heat of 130° to 140° C., the temperature in the interior had only risen to 35° C.

Cold.—Cold has practically no effect upon bacteria and their spores; alternately freezing at very low temperatures and subsequent thawing, however, destroys the vegetative form of some pathogenic bacteria. Cold has, accordingly, no place in the armamentarium of practical disinfection.

Direct Sunlight.—The chemically active blue, violet, and ultra-violet rays of the spectrum have a particularly strong germicidal effect upon both the vegetative forms and spores. The effect, however, is limited to the surface of objects. The beneficial effect of sunlight should not be underestimated, particularly in veterinary practice, where the great majority has not yet learned the importance of having stables, barns, and other places where animals are kept well lighted during the day and particularly well lighted and exposed to the sun after the prevalence of infectious diseases. Arloing noted the effect of sunlight upon anthrax bacilli and spores. Pansini showed that the bacilli in cultures are killed in from one to two and one-half hours; the moist spore directly exposed in one-half to two hours; the dried spores in from six to eight hours. Koch and others demonstrated the germicidal effect of sunlight upon tubercle bacilli. Rosenau found that the plague bacillus exposed to the direct action of the sunlight dies in half an hour, provided that the temperature is above 30° C. Even diffuse daylight, when acting long enough, has an inhibiting and detrimental effect upon many pathogenic bacteria, but it is very much slower than the effect of direct insolation.

Drying.—Some bacteria, like the glanders bacillus, the cholera spirillum, etc., are very rapidly killed by drying out, while others, like the tubercle bacillus, are only affected after they have been dried out for a long time. Certain spores, like those of anthrax, remain alive and virulent even after many years.

Electric Currents, X-rays, and Radium.—Electric currents, according to Zeit and others, have no direct effect upon bacteria, unless they are in solutions which give rise to detrimental electrolytic products, such as acids, ozone, etc. The *x*-rays, according to the same author, appear to have no germicidal effect. Radium emanations have an appreciable effect; they have killed anthrax spores after an exposure of three days. This effect, however, seems to depend entirely upon the easily absorbable rays and not upon the deeply penetrating rays, and is, therefore, only superficial.

The "Ideal" Disinfectant.—An ideal disinfectant would be one which, while highly efficient, deeply penetrating, and absolutely trustworthy, would not damage any of the objects to be disinfected. Such a disinfectant does not exist. Moist heat is excellent for cotton, wool, and linen, but it destroys leather, harness, etc. Formalin is strong and effective, does not easily damage, but its penetrating power is deficient. Some of the most important disinfectants extensively used in practical every-day work are the following:

Corrosive Sublimate, or Bichlorid of Mercury ($HgCl_2$).—This is one of the strongest antiseptics known, but its effectiveness is much decreased in the presence of albuminoids. This disadvantage can be partially corrected by adding *hydrochloric acid, tartaric acid, citric acid, chloride of sodium,* and other chemicals to the corrosive sublimate solutions. Bichlorid solutions are also objectionable in that they corrode metals, and are very poisonous. There is, however, hardly any better disinfectant for stables and barns which have been infected with anthrax bacilli and their spores. In this case the disinfectant should be used in the strength of 1 to 500, and it is, of course, necessary to proceed systematically so that all surfaces of walls and floors which may have become contaminated are exposed to the action of the solution. This may be accomplished by the use of mops or brooms or an elevated tank connected with a rubber hose, or best by the employment of a *pressure pump.* After thoroughly wetting every surface the solution should be allowed to act for from one to two hours, and then the place should be well cleaned with water. In the use of bichlorid solutions it must be remembered that they are very powerful poisons to man and domestic animals, and that subsequent removal by very thorough washing and flushing is necessary. Bichlorid cannot be used to disinfect tubercular material, such as sputum, caseous masses, etc., because the mercury salt forms a compound with albuminoids of very slight, if any, disinfectant value.

Caustic Lime.—Among other salts of the metals much used in practical disinfection, caustic lime deserves to be mentioned. Its effect depends upon its strongly alkaline reaction. On this account old solutions in which the lime salt has combined with carbon dioxide and formed carbonate of lime are of little or no value. The solution must, therefore, be prepared immediately before use. The following formula is recommended. Take a number of pounds of pure burnt lime (CaO), add slowly and gradually for each ten pounds $1\frac{1}{2}$ gallons of water, and finally add 20 gallons of water and mix well. This mixture will represent a 20 per cent. milk of lime which when fresh is quite an effective disinfectant.

Chlorinated Lime.—This is also known as chloride of lime, or *bleaching powder,* and is prepared by passing a stream of nascent chlorine gas over moist unslaked lime (calcium hydrate). It is a soft white friable substance, very slightly soluble in water, and of indefinite chemical composition. Its disinfecting value depends chiefly upon the amount of *calcium hypochloride* which it contains. Its bleaching and destructive properties limit its employment as a germicide, but it is used extensively in stables and barns for the disinfection of infected fecal matter, urine, manure, bedding, floors, and woodwork, etc.

Permanganate of Potash.—In a 4 per cent. solution this is a very powerful disinfectant and kills anthrax spores in about fifteen minutes.

Carbolic Acid.—Carbolic acid (C_6H_5OH) and other bodies belonging to this group of chemicals are very important disinfectants. They

are not as effective as corrosive sublimate, and have to be used in much stronger solutions when they are very poisonous; but they are quite penetrating, and their value is not decreased by the presence of albuminoid bodies, which gives them a wide range of application. Carbolic acid is generally used in 3 to 5 per cent. solutions. Its effect is very much increased by warming it to about 40° C., or by the addition of hydrochloric acid, but not by the addition of alcohol. Other bodies of the carbolic-acid group used as disinfectants are the *creosotes* (ortho-, meta-, and para-creosote, the mixture known as *tri-creosote*), *creolin*, and *lysol*. For the cleansing of woodwork, floors, walls, etc., Nocht's *carbol-soap solution* is highly recommended. It is prepared as follows: Dissolve 6 per cent. soft soap in hot water and add to the hot solution 5 per cent. of raw (100 per cent.) carbolic acid. Should any drops of tar form they should be removed and only the clear solution used. It does not stain and cleanses woodwork thoroughly.

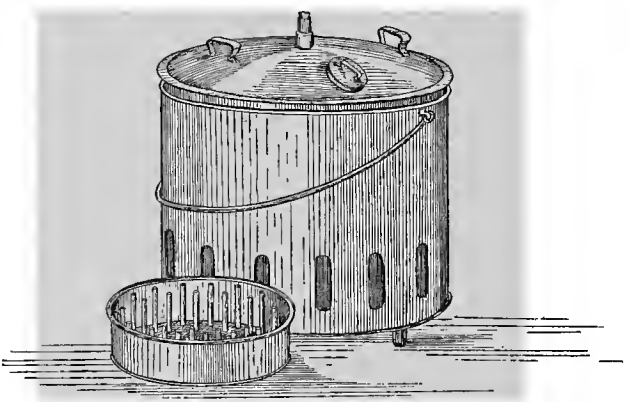
Formaldehyde.—Formaldehyde is the aldehyde[1] of methylic alcohol. It is a gaseous body of the chemical formula CHOH. It is found in commerce in the form of a watery solution, which should contain forty volumes of the gas per one volume of water. This solution in addition to being simply called formaldehyde is also known under a variety of proprietary names, such as *formol, formalin*, etc. When a solution of formaldehyde is warmed, and often merely upon standing undisturbed, a part of the gaseous body becomes *polymerized*, which means that several molecules unite to form a larger molecule. Generally three molecules of formaldehyde unite and form a *polymerization product* with the formula $(H—CHO)_3$. This body is known as *trioxymethylene, para-formaldehyde*, or simply as *paraform*. It is insoluble in water, and forms a white sediment in the vessel containing the formaldehyde solution. This chemical change is important, because such a white precipitate or sediment indicates that a formalin solution has lost much of its formaldehyde, that its value as a fluid or gaseous disinfectant has become weakened, and that it must be used afterward with a proper knowledge and consideration of this change. In making up formalin solutions it must always be remembered that the best commercial product contains not more than 40 per cent. of the disinfectant itself. Hence, if a 4 per cent. formalin solution is recommended for a certain procedure it must be prepared by taking *one part of formalin and nine parts of water*, not one part of formalin and twenty-five parts of water. Paraform is also used as a disinfectant, but according to a different method from that employed with the gaseous formaldehyde dissolved in water. The germicidal effect of a 4 per cent. formalin solution used as a disinfectant is about equivalent to a 1 to 1000 solution of corrosive

[1] An aldehyde is a body formed by the oxidation of a primary or secondary alcohol, that is, by the substitution of two or one H atom in the alcohol by one O atom.

sublimate and to a 5 per cent. solution of carbolic acid. The antiseptic value of formalin is very high, and it will inhibit the growth of bacteria if present in a proportion of 1 to 25 to 50,000; for germicidal purposes a $\frac{1}{4}$ per cent solution should be employed.

When formaldehyde was first introduced as a disinfectant it was used by allowing the gas to evaporate unaided from the watery solution. This is a slow process, requiring long exposures and large amounts of formalin to be in any way effective. Later, Trillat devised a method, using a lamp of his own construction, in which methyl-alcohol was evaporated under conditions that oxidized it into formaldehyde; but since the yield was only 7 to 8 per cent. of the methyl-alcohol used, this process was expensive, slow, and not very efficient. Other arrangements allow the evaporation of formalin with such additions (20 per cent. chloride of calcium-glycerin) that the formation of paraform is prevented. Still other devices are based upon the heating and decomposition of paraform into formaldehyde.

FIG. 108



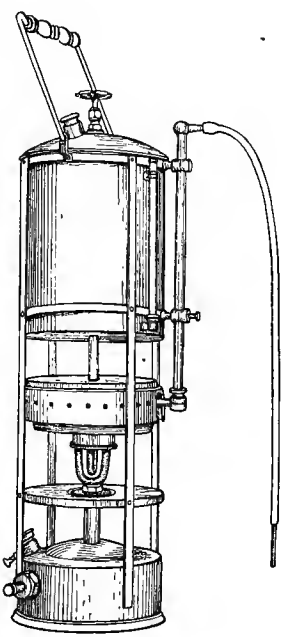
Breslau regenerator and lamp.

According to Gotschlich, the best formaldehyde disinfecting procedure is that of Flügge and his assistants. It is known as the *Breslau method*, and consists in evaporating a dilute formalin (1 part of formalin to 4 parts of water) in a simple apparatus. It has been found that such dilute formalin solutions do not upon evaporation form paraform, but allow all the formaldehyde present in solution to be expelled with the evaporating water. Harrington recommends very highly a formalin disinfecting apparatus devised by Professor Robinson, of Bowdoin College; also a regenerator made by the Sanitary Construction Company, which is said to be simple and economical. Paraform evaporation is brought about by the Schering paraform lamp.

Before using any of the apparatus mentioned, and formaldehyde

in the gaseous state in any form for the disinfection of the habitation of man and domestic animals, all doors, windows, cracks, and openings of any sort should be closed tight. In the case of barns, stables, etc., this task is not always easily accomplished. The amount of formalin necessary for the disinfection of any space depends upon its cubical contents. Approximately a pint of 40 per cent. formalin or 60 pastilles of paraform must be evaporated for each 1000 cubic feet of space. With the Breslau method about ½ pint for 1000 cubic feet is sufficient in a seven hours' exposure. Any formaldehyde remaining at the termination of disinfection may be neutralized by ammonia vapors. Formaldehyde is not an insecticide, and has no effect on these animals.

FIG. 109



Sanitary Construction Company's regenerator.

Sulphur Dioxide.—Besides formaldehyde, sulphur dioxide is the only other substance which is still extensively used as a gaseous disinfectant. It is produced by burning sulphur. The simplest and best arrangement is to place flowers of sulphur into an iron pot, which is placed in a second metal vessel containing a quantity of water. This arrangement reduces the danger of fire to a minimum, and by the heating and boiling of the water which will occur the amount of water vapor is supplied necessary to convert the sulphur dioxide into sulphurous acid, which is an effective germicide. Sulphur dioxide, in addition to destroying germs, also kills insects and vermin, such as mice and rats. Five pounds of sulphur should be burned for each 1000 cubic feet of space, and one pound of water should be vaporized for each five pounds of sulphur. Spaces to be disinfected by burning sulphur must, of course, be made as air-tight as possible.

Alcohol.—It should be remembered that absolute alcohol has practically no germicidal power. There is, however, considerable germicidal value in 50 per cent. alcohol, and it is well adapted for use in disinfecting surgeons' hands and the skin of animals before operations.

QUESTIONS.

1. What is meant by infected objects?
2. What does the term disinfection signify?
3. Can pathogenic microörganisms be removed from water without destroying them, and how?
4. What means different in type can be employed for disinfecting purposes?

5. How can the effect of a certain degree of heat upon pathogenic bacteria be ascertained?

6. What is meant by an inhibiting degree of temperature upon a bacterium? What by a germicidal effect?

7. What is the optimum, what the maximum temperature of a bacterium?

8. How is the effect of chemicals upon bacteria, both their antiseptic and their germicidal effect, ascertained?

9. What precautions are necessary to get trustworthy values for concentration and time?

10. What factors influence the effect of an antiseptic or disinfectant upon bacteria?

11. Describe the silk thread and the garnet method of testing germicidal values.

12. What is the difference between dry and moist heat as a germicide?

13. Give the effect upon the germicidal value of moist heat—of temperature, pressure, complete or partial saturation, admixture with antiseptics.

14. What is the effect of low temperatures upon pathogenic bacteria?

15. What is the effect of direct sunlight and diffuse light upon pathogenic bacteria?

16. Give some examples as to the effect on pathogenic bacteria.

17. What is the effect of diffuse daylight?

18. What is the effect of drying out bacteria?

19. What is the effect of electrical currents upon fluid cultures of pathogenic bacteria?

20. What is the effect of the *x*-rays?

21. What is the effect of radium emanations?

22. What are the properties of an ideal disinfectant? Name a number of them.

23. Describe the germicidal properties of corrosive sublimate in solution under varying conditions.

24. How should it be used in disinfecting a barn infected with anthrax bacilli and spores?

25. Describe the use of caustic lime as a disinfectant.

26. Describe the use of chlorinated lime as a disinfectant. When is it used in particular?

27. What is the effect of a 4 per cent. solution of permanganate of potash upon anthrax spores?

28. Give advantages and disadvantages of carbolic acid as a disinfectant.

29. What is an aldehyde? What is formaldehyde?

30. What is formol or formalin?

31. How is a 1 per cent. solution of formaldehyde prepared from a good commercial formalin?

32. What is meant by polymerization? What is the polymerization product of formalin? Is it a gas like the latter?

33. How can paraform be used as a disinfectant?

34. How does a 4 per cent. solution of formalin compare with corrosive sublimate and with carbolic acid?

35. What kind of an acid is carbolic acid?

36. How should formalin be used as a disinfectant? How much for each 1000 cubic feet of space?

37. What is the Breslau method of using formalin?

38. How is a space to be disinfected by formalin prepared?

39. How can the unpleasant pungent irritating smell of formalin be removed after disinfecting a space with it?

40. Discuss the value, advantages, and disadvantages of sulphur dioxide as a disinfectant.

41. How is it prepared? How much is required for each 1000 cubic feet of space?

42. Discuss the value of 100 per cent. and of 50 per cent. alcohol as a disinfectant.

43. What is the difference between disinfection and sterilization?

PART II.

SPECIAL BACTERIOLOGY.

CHAPTER XVI.

WOUND INFECTION, SUPPURATION, AND THE COMMON PYOGENIC BACTERIA.

Pyogenic Bacteria.—A large number of disease-producing bacteria, after having entered and multiplied in the body of man and the lower animals, may, under favorable conditions, lead to inflammatory reaction with the formation of pus. A limited number, however, are so frequently found as the cause of and associated with suppurative processes that they are called the pyogenic bacteria. The word pyogenic means pus-producing. The *pyogenic staphylococci* and *streptococci* are the most common of these. The former, particularly, are practically ubiquitous; they are found in the air, soil, water, and on the external and internal gastro-intestinal surfaces of man and animals. In the external world they ordinarily exist as saprophytes, and, as a rule, cannot enter the body through a healthy skin or mucous membrane. Absolutely clean wounds, such as are made by the surgeon with every possible aseptic precaution, will heal, as it is termed, by *primary (first) intention,* and will not suppurate. Wounds, however, which are received under natural conditions, will suppurate unless they are immediately cleansed with antiseptic solutions and dressed to exclude the air and other possible sources of contamination. The suppuration may vary in extent from being so scanty that it can only be recognized microscopically to being so great that there is a constant abundant discharge. The variations are dependent upon the species of the infecting pyogenic organism and upon the greater or lesser susceptibility of the infected race or individual. It is well known that certain species of animals are very susceptible to certain pyogenic organisms while others are entirely immune.

When pyogenic bacteria are confined to a certain locality the process is known as a *local infection.* It may be so severe that a sufficient quantity of toxins are absorbed from the infected focus to produce grave general symptoms, including more or less high fever. There

may be even more serious results when the bacteria enter the general circulation through the blood or lymph current. In this case their multiplication and the toxin production in the blood itself causes grave symptoms, generally including chills and severe fever of an irregular type. This process of pyogenic microbes multiplying in the general circulation is popularly known as *"blood poisoning,"* and technically as a *septicemia.*

Another pathologic process which may follow wound infection consists in the formation of small masses of bacteria, perhaps in combination with small flocculi of pus or bits of necrotic tissue. These are taken up by the circulation and carried along in the blood stream until they finally become lodged in parts at a considerable distance from the original focus of infection. In this manner the detached masses of bacteria may be transported to the lungs, heart, liver, kidneys, spleen, brain, or almost any one of the internal organs of the body from foci of infection on an extremity or some other place on the surface of the body. After they have finally become lodged, the bacteria multiply and may give rise to secondary and generally multiple foci of inflammation and suppuration. This pathologic occurrence or process is known as the formation of *multiple metastatic bacterial emboli* and the general condition of blood poisoning which has now become established as a *pyemia* or *septicopyemia.* It is more dangerous than a septicemia, and recovery is relatively rare.

When pus from an acute suppurative process is examined the causative bacteria are generally found in large numbers. This makes it comparatively easy to draw an accurate conclusion as to the nature of the particular infection. When more than one species of bacteria is present, either from the beginning or very soon after, the process is known as a *mixed infection.* An *acute* suppurative process due to pyogenic bacteria may develop into a *subacute* or *chronic* one. While the inflammation and suppuration may continue the infecting bacteria may die out and disappear. The pus then becomes void of living bacteria and is known as *sterile pus.* More or less extensive masses of necrotic tissue which are always a source of inflammatory irritation may, however, cause the inflammation and suppuration to continue. When necrotic tissue is present, it may become the soil for the development of purely saprophytic bacteria. *Gangrene* may set in, and if a sufficient quantity of the putrefactive products is absorbed a condition of *sapremia* may supervene.

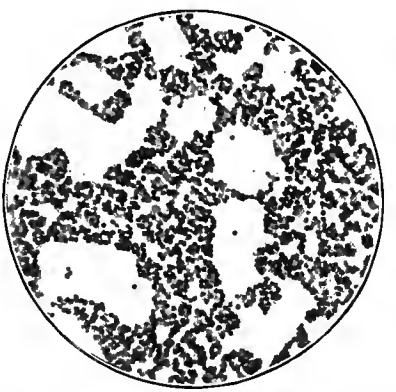
STAPHYLOCOCCUS PYOGENES.

The most common pus-producing bacteria in man and animals are the pyogenic staphylococci.

Varieties.—According to the pigments formed by these bacteria they are distinguished as:

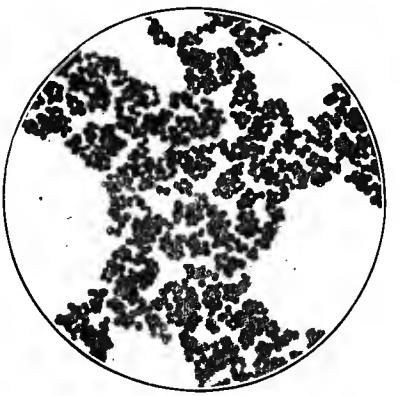
Staphylococcus pyogenes aureus (golden yellow pigment).
Staphylococcus pyogenes albus (white pigment).
Staphylococcus pyogenes citreus (lemon yellow pigment).
They are given in the order of their virulency, the aureus being the most, the citreus the least virulent.

FIG. 110



Staphylococcus pyogenes aureus. × 1000. (Author's preparation.)

FIG. 111



Staphylococcus pyogenes albus. × 1000. (Author's preparation.)

Occurrence and Pathogenesis.—Pyogenic staphylococci are found everywhere in the outside world, but are most numerous on the skin of man and animals. They are also frequently found in the feces. They vary much in virulency, and are most virulent when they come directly from a badly infected wound or a case of septicemia or pyemia. Surgeons operating on pus cases may easily and frequently do fatally infect themselves. The staphylococci, particularly the Staphylococcus pyogenes aureus, are the cause of all varieties of wound infections such as *septicemia, pyemia, endocarditis, septic pneumonia, puerperal fever, bone diseases,* etc.

Morphology and Staining Properties.—In the hanging drop the Staphylococcus pyogenes shows a completely globular or spherical shape. It exhibits a lively Brownian molecular motion. It stains well with the ordinary watery basic anilin dies. When not stained too deeply it frequently shows a line in the middle dividing it into two equal hemispheres. This line indicates the site where division will occur when the organism multiplies. The organism is Gram positive. The individual cocci vary from 0.7 to 0.9 of a micron in diameter, and sometimes have a diameter up to 1.2 micron. The Staphylococcus pyogenes albus and citreus are frequently larger than the aureus, but the size depends largely upon the culture medium. The cocci are generally smaller in young, rapidly growing cultures, and larger in old, slowly growing ones. In cover-glass preparations

from artificial cultures the organisms occur in large, irregular grape-like masses. In pus, however, this seldom occurs; instead they are generally found in small groups or pairs, double pairs (tetrads), or sometimes short chains. This sometimes makes it impossible to decide quickly whether the organism is a staphylococcus, a diplococcus, or a streptococcus. Cultures, of course, will definitely identify the organism. The staphylococci possess no flagella, and do not form spores.

Cultural and Biological Properties.—Pyogenic staphylococci grow on a great variety of artificial culture media. Their range of temperatures is wide and lies between 9° and 42° C.; the optimum temperature is at 24° to 28° C., not at blood temperature. They grow both in the presence and absence of oxygen, and also in a hydrogen atmosphere, but not in pure carbon dioxide or illuminating gas. The reaction of the culture soil, like the temperature, may vary considerably, but a slight alkalinity is most favorable to their growth. The organisms multiply very rapidly in *nutrient bouillon*, and under favorable conditions a tube inoculated in the ordinary manner may, after twenty-four hours, contain 85,000,000 cocci per cubic centimeter. The bouillon soon becomes intensely clouded, sometimes a slight pellicle develops on the surface, and a slimy sediment is always formed at the bottom of the tube after a growth of several days. The tubes have a strong smell of old starch paste. The organism also grows well in Dunham's *peptone water* and in *milk*. The latter shows coagulation sometimes after a few days, always after eight days. In *gelatin stab cultures* the growth extends along the entire stab, and after a few days liquefaction begins at the surface and progresses downward. On *gelatin plates* small yellow points around which the medium is liquefied appear after two days. On *blood serum* and Loeffler's *blood-serum mixture* the growth liquefies the medium very slowly, sometimes not at all. If *fresh sterile blood*, particularly rabbit's blood, is added to a solid culture medium on which the staphylococcus is subsequently grown, solution of the red blood corpuscles (*i.e.*, hemolysis) occurs. *Potatoes* are a good culture medium for the organism, which does not ferment sugar.

Pigment formation requires the presence of oxygen. It is not well marked in the presence of too much strong diffuse light, but shows best when the cultures receive only little light and are kept at temperatures between 20° to 22° C.

The *liquefying properties* of the staphylococci depend upon the secretion of a *tryptic ferment*. It is now claimed that the strong ferment liquefying gelatin and the rather weak one liquefying blood serum and egg-albumen are two distinct types. Hemolysis due to the growth of the organism is caused by a hemolysin. Staphylococci also produce a substance which is very injurious to the leukocytes of the rabbit. This body has been called *leukocidin*, which means killing white blood corpuscles. Several investigators claim

to have been able to produce *amyloid substance*[1] in the internal organs of animals treated with prolonged systemic infections of pure cultures of staphylococci.

Experiments with Staphylococci.—Many investigators have experimented upon themselves with staphylococci and animal experiments without number have been made. The results show that man is more susceptible to the detrimental effects than any of the lower animals. The difference in virulency in the Staphylococcus pyogenes aureus, albus, and citreus can be very well exhibited by injecting small amounts of bouillon cultures into the anterior chamber of the eye of a number of rabbits. The aureus generally causes a very violent suppurative panophthalmitis (inflammation of the entire eyeball), destroying the eye, and sometimes leads to a general infection (septicopyemia) and death. The albus produces a less serious inflammation and the citreus a very mild one.

Resistance of the Organisms.—The resistance of different strains of staphylococci toward inimical, physical, and chemical agents varies considerably. Sternberg killed cultures by an exposure to a temperature of 62° C. for ten minutes and 80° C. for 1½ minutes. Others have had cultures which could withstand 60° C. for one hour without being killed. The resistance of these cocci evidently is increased by previous drying out, particularly in pus. An exposure for thirty to sixty minutes at 80° C., however, apparently kills the pathogenic staphylococci under all conditions. Repeated freezing alternating with thawing seems to have no effect whatever upon the organism. Direct sunlight does not appear to kill the staphylococci even after several hours. Drying has little effect, and kills them only after many weeks. The effect of antiseptics and germicides upon pyogenic bacteria is as follows: Solution of corrosive sublimate 1 to 1000 kills the staphylococci in thirty to sixty minutes; chloroform vapors in twenty minutes; iodoform has no effect whatever; absolute alcohol has no effect upon dried cocci, but 50 per cent. alcohol kills them in ten minutes; 3 per cent. carbolic acid in two to two and one-half minutes; 1 per cent. solution of formalin in twenty-four hours; even a 5 per cent. solution of formalin only kills after thirty to thirty-five minutes. Some of the anilin stains, like methyl violet, even in very weak solutions, kill staphylococci very quickly.

Vaccine Therapy.—Chronic staphylococcus infections of a low type, such as furuncles, discharging sinuses, old abscesses, etc., have been found capable of improvement, and often of cure, by vaccine or bacterine treatment. An autogenous vaccine, *i. e.*, one from a pure culture obtained from the infected patient, gives the best results in these

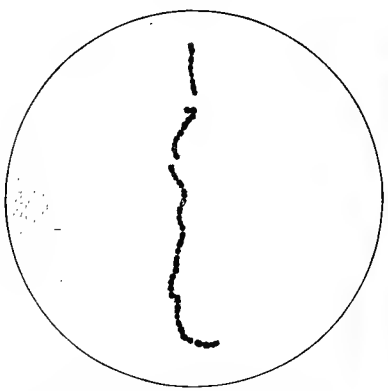
[1] Amyloid material or substance is one of the hyaline materials, which are degenerative, pathologic products. It has certain characteristic staining properties and certain color reactions like starch (amylum). It is, however, not a carbohydrate like starch, but a proteid body. It is first formed or deposited in the walls of the small vessels of the internal organs, particularly the spleen, liver, and kidneys.

cases. The killed staphylococci are injected in proper doses at intervals of about six to eight days. If it is too troublesome to prepare an autogenous vaccine, a stock vaccine, as prepared by pharmaceutical houses, may be used.

STREPTOCOCCUS PYOGENES.

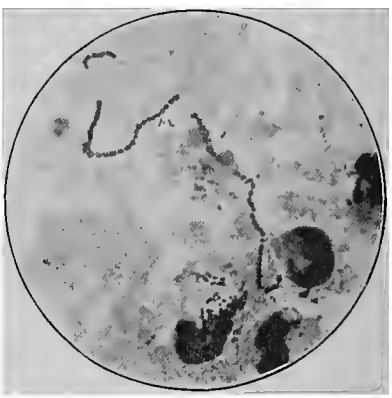
Occurrence and Pathogenesis.—Pyogenic streptococci are evidently not found as commonly in the outside world as staphylococci, nor do they appear to thrive as well as saprophytes. The Streptococcus pyogenes is the cause of suppurative processes of all kinds, such as *septicemia, pyemia, puerperal infection, erysipelas,* etc. General infections by the Streptococcus pyogenes are, as a rule, even more virulent than those of the staphylococcus.

Fig. 112



Streptococci from a pure culture in bouillon. × 1000. (Kolle and Wassermann.)

Fig. 113



Streptococci from human pus, Gram's stain. × 1000. (Author's preparation.)

Morphology and Staining Properties.—The Streptococcus pyogenes is non-motile, has no flagella, does not form spores, and can grow in the presence or absence of oxygen. The individual cocci vary from 0.4 to 1 micron in diameter. The length of the chains differs so much that varieties such as the Streptococcus pyogenes longus and the Streptococcus pyogenes brevis, the long and the short chain cocci, have been distinguished. The individual cocci forming the chain vary not only in size but in shape. Some are entirely spherical; others are flattened at both poles, so that the chain seems to consist of disk-like bodies; still others are flattened out laterally, making the individual cocci oval or like bacilli with pointed ends. When the cocci grow in the long axis of the chain with infrequent division, chains are formed which look much like streptobacilli, *i.e.*, bacilli in the form of chains. The formation of short or long chains is not an absolutely

permanent characteristic of certain varieties. It often depends upon differences in occurrence, environment, and culture media. Certain stems of streptococci, however, generally show a tendency to form short chains of only four, six, or eight cocci, while others have the opposite tendency. Virulent forms in tissues often appear in short chains; these generally have a capsule. The Streptococcus pyogenes stains well with the ordinary watery anilin dyes, and keeps Gram's stain. There are, however, other pathogenic streptococci, such as the streptococcus found in abscess of the udder in cows (Nocard's streptococcus), that are Gram negative, as are also some saprophytic streptococci.

Cultural and Biologic Properties.—Pyogenic streptococci grow and exhibit the typical chain form much better in fluid than in solid culture media. The Streptococcus pyogenes longus clouds the *bouillon* diffusely; the Streptococcus pyogenes brevis produces less clouding. A faintly alkaline reaction of the nutrient bouillon is best. An increase of the peptone of the bouillon from 1 to 3 to 5 per cent. and the addition of 0.2 to 1 per cent. glucose is also favorable to the growth of the organism. Too much glucose, however, lessens the virulency of the streptococcus and causes it to die out sooner, since the acid formed from the sugar changes the reaction of the culture soil.

Blood serum, generally used with the addition of one part of nutrient bouillon to three parts of serum, is an excellent medium for the growth of pyogenic streptococci with preservation of their virulency. Human, rabbit, or horse serum may be used. The serum must not be solidified, as the organism does not grow very abundantly on solid media. On *agar* plates kept in the incubator, small grayish or yellowish gray, finely granular colonies develop, which generally do not exceed 0.5 mm. in size. The deep colonies are brownish, round, or oval. *Agar streak* and *stick cultures* are not characteristic. *Gelatin* kept at 20° to 22° C. is generally not liquefied, but gelatin which can be kept at 29° C. is sometimes. Saprophytic streptococci, cultivated from dust and from the contents of the intestines, liquefy gelatin. Pyogenic streptococci only occasionally grow on *potatoes*. The *optimum temperature* of growth is in the neighborhood of the blood temperature. At 12° to 15° C. the development is poor, at 24° C. good, and best at 35° to 37° C.; at 40.5° C. it falls off and at 42.3° C. it ceases entirely. Slightly different figures are given by other authors. Streptococci can grow in the presence *of oxygen*, but they do not require it, and in fact some varieties evidently grow better *anaërobically*. All streptococci form acid in their growth, chiefly lactic acid, and coagulate *milk*, in which they generally grow poorly because of the acid formation. Some stems form a brownish-yellow pigment, particularly in gelatin and in the sediment of bouillon cultures. While pyogenic streptococci generally grow for two to three days only on artificial culture media and then die out, certain stems may remain alive longer, occasionally for several weeks.

Resistance.—The Streptococcus pyogenes generally dies quickly in liquid cultures or when dried out on silk threads. When contained in dry pus it sometimes remains alive for weeks and months, particularly if the cocci have dried out slowly and are subsequently kept at low temperatures and protected against light. They are comparatively resistant against high temperatures, generally being able to withstand heat of 60° C. for one hour and occasionally for two hours; 70° to 75° C. acting for one hour, however, positively kills them. Cold appears to have no effect. A number of antiseptics have been tried by von Lingelsheim. He gives the following concentrations as killing Streptococcus pyogenes in fifteen minutes:

Hydrochloric acid	1 to 150
Sulphuric acid	1 to 150
Ammonia	1 to 15
Corrosive sublimate	1 to 1500
Copper sulphate	1 to 125
Chloride of iron	1 to 350
Carbolic acid	1 to 200
Kresol	1 to 175
Lysol	1 to 200
Kreolin	1 to 80

Vaccine Therapy.—This appears to be valueless in the acute infections, but in slow chronic cases the bacterine treatment often leads to good results.

BACILLUS PYOCYANEUS.

Occurrence and Pathogenesis.—The Bacillus pyocyaneus was first found in and isolated from green pus. It is, however, also very common as a saprophyte in the outside world. It has been found in sewage, manure, water, the intestinal contents of man and domestic animals (particularly the hog), rooms and hospital wards, barns, etc. It generally lives as a harmless commensale in the intestines of man and animals, but it has been known in ill-nourished, weak children to invade the organism from the intestine and in a few cases to have led to a general septicemia and death. It is frequently the cause of *local suppurations*, also of *purulent middle-ear inflammations*. It imparts a green color to the pus. The Bacillus pyocyaneus is also found in pus in animals, but it is doubtful whether it alone can start a suppurative inflammation in domestic animals. According to Baruchelli, injection of the Bacillus pyocyaneus into the peritoneal cavity of a male guinea-pig occasionally produces a *periorchitis* which may cause it to be confounded with the Bacillus mallei in *Strauss' biologic test for glanders* (see Chapter XXVI on the Glanders Bacillus).

Morphology and Staining Properties.—The Bacillus pyocyaneus is generally a small, slender bacillus, but it also occurs in larger, plumper varieties and the measurements given by various authors are from 0.3 to 1 to 0.6 to 2 to 6 micra. It has rounded ends and often forms short chains of a few individual bacilli; longer pseudofilaments are rare. The bacillus is very actively motile and possesses a flagellum

at one end. After long-continued culture on artificial media the flagellum may be lost, but it reappears after inoculation into an animal. It does not form spores. It stains well with the watery anilin dyes, but is Gram negative.

Cultural and Biologic Properties.—The Bacillus pyocyaneus grows well at room and incubator temperature. On *gelatin plates* small yellowish-white colonies appear first in the deeper parts and extend rapidly toward the surface, where they then show a dark yellow centre and a periphery with radial striation. The medium itself assumes a typical greenish fluorescent tint around the colonies. The gelatin is liquefied and the culture sinks to the bottom, forming a slimy red-brownish mass. In *gelatin stick cultures* liquefaction first appears at the surface in a funnel-shaped manner, and then rapidly spreads downward. On *agar slants* kept at incubator temperature the growth is rapid, and the green pigment generally turns brownish after two days, spreading successively through the entire culture medium. *Pigment formation* occurs only in the presence of oxygen, and the liveliest colors are formed at room, but not at incubator, temperatures. The bacillus not infrequently produces a blue or even from the beginning a greenish-brown pigment instead of a decided green color.

Fig. 114

Bacillus pyocyaneus. × 1000. (Author's preparation.)

In *bouillon* the bacillus grows well. A white ring first shows at the margin of the free surface, and from it a complete pellicle is formed. From the latter a green zone extends downward into the medium. After two weeks the whole growth sinks to the bottom and forms a slimy sediment. After many weeks in the incubator the formation of an *autolytic ferment* causes the growth to undergo *self-digestion*. *Milk* is coagulated by the bacillus. The organism grows well on *potatoes*, and forms first a grayish-brown and later on a yellowish-green pigment, which is composed of two constituent bodies. One of these, known as *pyocyanin*, is bluish green and soluble in chloroform, the other is greenish, fluorescent, and insoluble in chloroform or alcohol, but soluble in water. Pyocyanin is originally a colorless substance, and obtains its color only after subsequent oxidation.

Toxins.—The Bacillus pyocyaneus is pathogenic in experimental inoculation for guinea-pigs and goats; rabbits, mice, and pigeons are slightly susceptible. Wassermann has shown that the pathogenic effect of the organism upon man and animals depends upon a *soluble toxin* and an *insoluble endotoxin*.

Resistance.—The Bacillus pyocyaneus is quite resistant. It cannot be readily killed by drying out, and its behavior toward antiseptics and germicides is similar to that of the pyogenic staphylococci.

QUESTIONS.

1. Explain the term pyogenic bacteria.
2. Which are the most common pyogenic microörganisms?
3. Where are they found?
4. What does the term ubiquitous mean?
5. Are pyogenic bacteria most commonly found as parasites?
6. What is meant by wound healing by primary intention?
7. What is meant by a local; what by a general pyogenic infection?
8. What is a septicemia?
9. What are multiple metastatic bacterial emboli?
10. What is a septicopyemia?
11. What is a mixed infection?
12. What is a sapremia?
13. Name the three different varieties of the common pus-forming cocci?
14. Name a number of diseases caused by the pyogenic staphylococci.
15. Describe the Staphylococcus pyogenes aureus in a hanging drop and in a stained cover-glass preparation.
16. How does this coccus act when stained by Gram's method? Describe this staining method.
17. How does the Staphylococcus pyogenes generally present itself in pus?
18. At what temperature does this coccus grow? What is its optimum temperature? What is its action toward free oxygen?
19. Describe a bouillon culture and a gelatin stab culture of the Staphylococcus pyogenes aureus.
20. What is meant by the hemolytic property of the Staphylococcus pyogenes?
21. What is leucocidin?
22. What is amyloid material or substance?
23. Discuss the resistance of the Staphylococcus pyogenes toward physical and chemical agencies.
24. What is the effect of vaccine therapy in chronic staphylococcus infection? Describe the principle and details of staphylococcus vaccine therapy.
25. Give the morphologic features of the Streptococcus pyogenes.
26. What is the difference between Streptococcus pyogenes longus and Streptococcus pyogenes brevis?
27. What are the staining properties of the Streptococcus pyogenes?
28. Name some Gram-negative streptococci.
29. What kind of media are best adapted to the growth of Streptococcus pyogenes?
30. What effect have peptone and sugar upon the growth of the Streptococcus pyogenes?
31. What culture medium is best for preserving the virulency of Streptococcus pyogenes?
32. Describe the colonies of the streptococcus on agar plates.
33. Under what conditions does the streptococcus liquefy gelatin?
34. What is its optimum temperature? What is its relation to free oxygen?
35. Discuss the resistance of the streptococcus.
36. Describe the occurrence and pathogenesis of the Bacillus pyocyaneus. Why is it called pyocyaneus?
37. Describe the morphologic features of this bacillus.
38. Does it belong to the amphitrichæ or peritrichæ?
39. What effect does an intraperitoneal injection of the bacillus into a male guinea-pig sometimes have? Why is it important to remember this?
40. Describe a gelatin plate and a gelatin stick culture of the organism.
41. Describe a bouillon culture.
42. What is meant by autolysis and an autolytic ferment secreted by the Bacillus pyocyaneus?
43. What are the properties of the two pigments formed by the Bacillus pyocyaneus?
44. What kind of toxins does this bacillus form?
45. Discuss the resistance of the organism.

CHAPTER XVII.

PYOGENIC BACTERIA IN DOMESTIC ANIMALS—STREPTOCOCCUS EQUI—STREPTOCOCCI IN MORBUS, MACULOSUS EQUI, AND PLEUROPNEUMONIA IN HORSES—BOTRYOCOCCUS ASCOFORMANS—PYOGENIC BACTERIA IN CATTLE—BACILLUS PYOGENES SUIS.

THE common pyogenic bacteria described in the preceding chapter are in man, in the majority of cases, the cause of suppurative inflammations, septicemia and pyemia. They are also very frequently responsible for the identical pathological processes in domestic animals. Karlinski investigated a large number of pyogenic affections in man and animals and found the various causative bacteria as follows:

		Cases.
In man	Streptococci	45
In man	Staphylococci	144
In man	Other bacteria	15
In mammals	Streptococci	23
In mammals	Staphylococci	45
In mammals	Other bacteria	15
In birds	Streptococci	11
In birds	Staphylococci	40
In birds	Other bacteria	20

Lucet made a bacteriological examination of 93 cases of suppurations of various types in horses and found staphylococci in 86 cases. The three varieties were found either in pure or mixed cultures, but the Staphylococcus pyogenes albus appeared more commonly as the cause of suppuration in the horse than the Staphylococcus pyogenes aureus. In man the contrary is true. Schütz, Jensen, and Nocard similarly found staphylococci in most cases of suppuration in the horse, and Streptococcus pyogenes also occurred in a number of cases. Pyogenic staphylococci and streptococci have also been found in *pyogenic, metastatic joint affections in horses,* particularly when young; occasionally as the cause of *suppuration and erysipelas in cattle,* and also in *puerperal fever in cows.* Ordinarily, cattle do not seem very susceptible to infection by these pyogenic bacteria. Dogs, however, are more susceptible. Streptococcus pyogenes has further been found as the cause of obstinate eczema of the tail of the horse.

A number of bacteria acting as specific pyogenic microbes for certain domestic animals will now be considered briefly.

STREPTOCOCCUS EQUI.

Occurrence and Pathogenesis.—The Streptococcus equi was first seen by Rivolta in 1873, and identified as the cause of *strangles in the horse* by Schütz in 1888, and also by Sand and Jensen, independently, at about the same period. The specific disease which it causes is also known as *Coryza contagiosa equorum, distemper; "Druse der Pferde,"* in German; and *"Gourme,"* in French. Strangles is a febrile, infectious, mucopurulent affection of the nasal and buccal mucosa, with abscess formation in the laryngeal and retropharyngeal lymph glands. The Streptococcus equi is found in the nasal discharge and in the abscesses in the affected lymph glands; in the latter often in pure culture, in the former, of course, mixed with other bacteria. It sometimes causes a simple nasal catarrh without suppurative processes in the glands, and at other times *suppurative pleuritis* and *exanthematous affections,* with *vesicle* and *pustule* formation. It may also cause *septicemia* and *pyemia.* Metastases have been found in the brain, liver, spleen, kidneys, mesenteric glands, and intestines, etc.

Morphology and Staining Properties.—The Streptococcus equi is composed of cocci which may be spherical or oval or perfectly cylindrical short disks. The latter arrangement is often seen in tissues, and the disks may be so crowded that the dividing lines of the individual cocci are invisible, causing the organism to appear like a curved filament rather than a true streptococcus. The chains are usually very long, and may contain from fifty to one hundred cocci; they are rarely straight, but, as a rule, curved and twisted. Short chains also occur, and sometimes even diplococci and single cocci. The organism stains with the ordinary watery anilin stains, and is Gram positive. The washing in alcohol, however, must not be continued for too long a period, as it may cause decolorization of the organism. Some observers claim that the Streptococcus equi does not stain by Gram's method.

Cultural and Biologic Properties.—The Streptococcus equi grows in the presence or absence of free oxygen, and at room or incubator temperature. In *bouillon* the growth forms fine flocculi, which fall to the bottom of the tube and later develop a sediment, leaving the upper strata of the medium clear. In *gelatin stick cultures* very small punctiform white colonies are formed along the stab. On *agar slants* or *plates* the colonies reach the size of a pinhead. They are grayish, not transparent, do not become confluent, and adhere firmly to the medium. On solidified *blood serum* the colonies are glassy and translucent, and become confluent at a later stage. In the condensed water of blood-serum tubes a fine precipitate composed of very long chains is found. Some authors claim that there is no growth on *potatoes,* others state that a grayish, slimy growth is present after eight days. The organism does not ferment sugar; on artificial media

it sometimes forms pseudofilaments. It is pathogenic to mice, and according to Rabe, also to guinea-pigs.

STREPTOCOCCI IN OTHER DISEASES.

Streptococci in Morbus Maculosus Equorum.—The disease known as morbus maculosus equorum, acute hemorrhagic anasarcous toxemia, or petechial fever, occurs either sporadically or in epidemic form, particularly after influenza and strangles. Lignière, who investigated the bacteriology, claims that he generally found the Streptococcus pyogenes and more rarely the Streptococcus equi and the Bacillus equisepticus in the blood of horses which had died from the disease. However, neither Lignière nor any other investigator has been able to produce the disease experimentally by the inoculation of these organisms.

Streptococci in Contagious Pleuropneumonia of Horses.—An organism which presents itself as a *diplococcus* in tissues, but as a *streptococcus* in pure cultures, was described as the cause of this disease by Schütz in 1887. It often shows a capsule in tissues, stains with the ordinary watery anilin stains, but not by Gram's method. It grows on *agar* and *gelatin* at room and incubator temperature, and Schütz claims that he has been able to produce the disease in horses by intrathoracic injections of pure culture. According to Lignière, Schütz's organism is identical with the Streptococcus equi.

Streptococci in Apoplectiform Septicemia in Chickens.—Noergaard and Mohler have described a streptococcus occurring in short or long chains, with individual cocci 0.6 to 0.8 micron in diameter, as the etiologic factor of this disease. The organism is Gram positive. It grows in the presence or absence of oxygen. It forms flaky masses in *bouillon*, and leaves the fluid clear, changes an alkaline medium to an acid one, and does *not liquefy gelatin*. It does not greatly change the appearance of *milk*, nor does it form a visible growth on *potatoes*. It is fatal to fowls, mice, rabbits, and swine, but not to guinea-pigs, dogs, and sheep.

BOTRYOCOCCUS ASCOFORMANS.

Occurrence and Pathogenesis.—The organism, now generally known as Botryococcus ascoformans, is the cause of a suppurative infection known as botryomycosis. The clinical manifestations and histopathology of the disease somewhat resemble actinomycosis, but the causative organism is entirely distinct. While actinomycosis is common in cattle and rare in the horse, botryomycosis is common in the horse and relatively rare in cattle. It is also occasionally seen in the hog and in man. The disease is remarkable because it represents a pathologic process showing some common features of an infectious granuloma and of a true tumor. The botryomycotic new formations

represent grayish-white, fibrous, lardaceous connective-tissue masses in which smaller areas of cellular vascular granulation tissue are found, often with small cavities and fistulous tracts containing varying amounts of pus, and in the latter the peculiar *zoögleal masses* of the Micrococcus ascoformans. Botryomycosis is nearly always a wound infection. It may originate at any break in the surface made by the harness, and it frequently begins in castration wounds. The process extends from the cutaneous and subcutaneous tissue into the lymph glands and muscles, where it may lead to a botryomycotic myositis. In castration wounds the botryomycotic masses along the seminal cords sometimes assume a large size and a mushroom shape. Botryomycotic tumors of the chest and shoulders of the horse weighing from fifty to one hundred pounds have been reported. Tumors of smaller size have also been found at the lips, conchæ of the ears, mammary glands, anus, tail, etc.

Morphology and Staining Properties.—The zoögleal masses found in the pus and the tissues can be seen with the naked eye as pinhead-sized, yellowish-white bodies. When examined microscopically they appear mulberry-shaped, and are found to consist of large, densely crowded cocci. They are contained in and surrounded by a mass of protoplasm, and for this reason are known as zoögleal masses. The protoplasmic envelope or capsule is the thicker the larger the colony of cocci. The individual cocci are comparatively large, *i. e.*, 1 to 1.5 micra in diameter. They stain best with anilin-water-gentian violet.

Cultural and Biologic Properties.—The Botryococcus ascoformans can be easily cultivated on *gelatin* and *potatoes*, less easily on *agar*. It liquefies gelatin. The artificial cultures never show the zoögleal masses, which are seen in the infected tissues and pus. In pure cultures the organism closely resembles the Staphylococcus pyogenes aureus, but the preponderance of evidence is that the botryococcus is a distinct organism and not a variety. While it is true that cultures of the botryococcus, when inoculated into a horse, may produce either a simple suppuration or a botryomycosis, injections of the Staphylococcus pyogenes aureus into any animal have never been known to produce a botryomycosis.

Experimental Infection.—Guinea-pigs after inoculation with the Botryococcus ascoformans die from septicemia. In sheep and goats subcutaneous infection produces an edema, sometimes with necrosis of the skin, and occasionally followed by death. Kitt reports that pigeons and ducks die after inoculations.

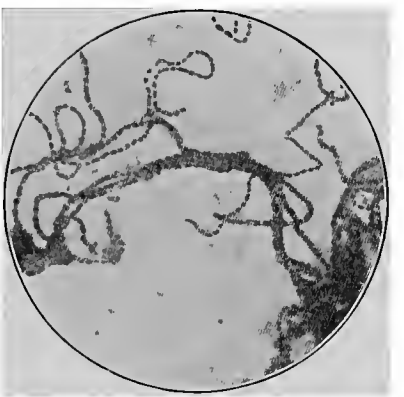
PYOGENIC BACTERIA OF CATTLE.

Suppuration in cattle in a majority of cases is not due to the common staphylococci so frequently found in man and the horse, but to the following special bacteria:

Streptococcus pyogenes bovis.
Staphylococcus pyogenes bovis.
Bacillus pyogenes bovis.
Bacillus liquefaciens pyogenes bovis.
Bacillus crassus pyogenes bovis.

Streptococcus Pyogenes Bovis.—This organism appears as small cocci arranged in long chains; they grow particularly long in nutrient bouillon. It does not liquefy gelatin, nor grow on potatoes. Bouillon first becomes cloudy, later a scanty sediment forms on the bottom and the fluid again becomes clear. It is not pathogenic to guinea-pigs and rabbits.

FIG. 115



Streptococcus pyogenes bovis, pure culture obtained from the uterus of a cow. × 1000. (From preparation of Dr. L. E. Day.)

The Staphylococcus Pyogenes Bovis.—The organism is smaller than the Staphylococcus pyogenes aureus of man and the horse. It does not grow well in artificial cultures, and soon dies out. It does *not liquefy gelatin*, while the Staphylococcus pyogenes aureus does. Cultures on artificial media show a very low degree of virulency.

BACILLUS PYELONEPHRITIDIS BOVIS.

This bacillus is an important pus producer in cattle, frequently causing a pyelonephritis, known as *pyelonephritis bacillosa bovum.* The disease is generally observed in cows shortly after parturition, particularly in cases of retention of the placenta. Occasionally pyelonephritis in cows is also caused by the common staphylococcus and by the Bacillus pyocyaneus.

Pathologic Lesions.—Pyelonephritis bacillosa bovum is an inflammatory, purulent, or diphtheritic necrotic process, characterized by great enlargement of one or both kidneys, with enlargement of the

pelves and ureters. The latter contain a dirty grayish or brownish purulent, bloody fluid. The urine found in the bladder is likewise purulent and hemorrhagic. The mucosa of the pelvis, ureter, and bladder are covered with a thick tenacious pus and often with necrotic diphtheritic masses. The disease is generally chronic, rarely acute, and always terminates fatally.

Morphology and Staining Properties.—The specific bacilli are from 2 to 3.8 micra long and from 0.6 to 0.7 of a micron wide. They are slender, generally slightly curved, non-motile, and do not form spores. They stain with the watery basic anilin stains and are Gram positive. They sometimes show polar granules. They may be club-shaped, and occasionally form branches.

Cultural and Biologic Properties.—They are strictly aërobic and will not grow on artificial culture media in the absence of oxygen. They grow readily on *agar* and *blood serum* at room temperature, better in the incubator at 37° C., and form small grayish-white punctate colonies with a sharp margin. In bouillon they form a fine sediment after two days, the supernatant fluid remaining clear. They grow neither in milk nor on potatoes, and generally not in gelatin. The cultures have a tendency to die out quickly.

The bacillus is not pathogenic to man, but it sometimes leads to suppuration when injected into mice and guinea-pigs. Intravenous injection in cattle after ligation of the ureter produces a typical attack of pyelonephritis bovis. The natural mode of infection in cows is through the genito-urinary tract after parturition. The organism may also enter the kidneys through the blood current (hematogenous infection).

BACILLUS PYOGENES SUIS.

Occurrence and Pathogenesis.—Suppuration in hogs is generally caused by a specific microörganism known as the Bacillus pyogenes suis. Inflammatory suppurative processes, generally confined to the serous membranes, particularly the pleura, pericardium, and peritoneum, are frequently seen in hogs after they are slaughtered. The membranes are thickened by inflammatory connective tissue, and excrescences in the form of more or less spherical or elliptical masses project beyond their surface. These masses are, as a matter of fact, small abscesses surrounded by a capsule of tough, fibrous, connective tissue. If incised, the abscesses discharge a thick, tenacious, yellowish-green or greenish non-fetid pus. In advanced cases abscesses are also found in the lymph nodes of the thorax, head, and muscles. Occasionally they are found over the entire body, in all the internal organs.

Morphology and Staining Properties.—The microscopic examination of pus from hogs shows a short, slender, non-motile bacillus, often present in very large numbers. It stains best with anilin-water gentian violet or carbol-fuchsin; it is Gram negative and is not acid

fast. In old abscesses the bacilli present involution forms and stain poorly.

Cultural and Biologic Properties.—The Bacillus pyogenes suis grows best *on coagulated blood serum* at blood temperature in the incubator. It also grows on *agar*, in *gelatin stick* cultures, and in *bouillon*. The latter remains clear, or becomes very slightly cloudy. A whitish sediment collects at the bottom of the tube. On blood *serum* whitish, delicate, dry, punctate colonies, which liquefy the culture soil slightly, develop after several days. On *potatoes* a similar growth occurs. No gas is formed in glucose agar.

Experimental Inoculation.—The Bacillus pyogenes suis is pathogenic to rabbits and mice when injected into the peritoneal cavity. It produces a purulent peritonitis, with a tendency to encapsulated abscess formation, resembling that occurring in the hog. In hogs the natural mode of infection is by inhalation or through wounds, such as castration wounds, injuries in the mouth, umbilical cord, etc.

QUESTIONS.

1. What organism is most commonly the cause of suppuration in horses?
2. What other organism causing suppuration in man causes a similar process in the horse?
3. What organism causes strangles in the horse? Under what other names is this infection known?
4. Where is the Streptococcus equi found in strangles and what other equine diseases does it sometimes cause?
5. Describe the morphology of the Streptococcus equi.
6. What are its staining properties?
7. Describe a bouillon culture of the Streptococcus equi.
8. Also a growth on agar, gelatin, and blood serum.
9. What animals are susceptible to infection with the Streptococcus equi?
10. What bacteria have been found in the blood of horses dead from Morbus maculosus?
11. Describe the organism found by Schütz in contagious pleuropneumonia of horses.
12. Describe the organisms found as the cause of apoplectiform septicemia in chickens.
13. What kind of disease is botryomycosis? What is its etiologic factor?
14. Why is botryomycosis in some of its features like tumor formation?
15. Describe the seat and appearance of botryomycotic lesions.
16. Describe the Botryococcus ascoformans as seen in pus both with the naked eye and with the microscope.
17. What is a zoögleal mass? how is its formation brought about? How does it look in a pure culture of Botryococcus ascoformans?
18. How does the organism look in pure culture on agar?
19. For what animals is the organism pathogenic?
20. Under what conditions does the Staphylococcus pyogenes aureus produce botryomycosis?
21. Name the bacteria which are generally the causes of suppuration in cattle.
22. What is generally the cause of pyelonephritis in cattle? Describe the organism and the pathogenic changes which it produces.
23. What are the cultural properties of this organism?
24. What is a hematogenous infection?
25. What organism generally produces suppuration in hogs? Describe its morphology.
26. Describe the abscesses due to this organism.
27. Describe the cultural properties of the Bacillus pyogenes suis.
28. What animals are susceptible to it?

CHAPTER XVIII.

BACTERIA PRODUCING DIPHTHERITIC INFLAMMATIONS—BACILLUS DIPHTHERIÆ—BACILLUS NECROPHORUS—BACILLUS DIPHTHERIÆ AVIUM.

A NUMBER of anatomical types of inflammation, such as serous, fibrinous, purulent, hemorrhagic, and diphtheritic, are distinguished in pathology. The microörganisms most commonly causing purulent inflammations have been described in the two preceding chapters. Some of the most important organisms causing diphtheritic inflammations will now be considered. A diphtheritic inflammation is one marked by extensive necrosis, either due in the beginning to chemical or physical influences (acids, alkalies, heat, cold), or resulting, at an early stage, from the toxins of the pathogenic invading and multiplying organisms. In either case an area of tissue containing numerous necrotic cells is formed; generally on the surface adjacent to the necrotic zone there is a hyperemic one with dilated vessels, with an abundant transudate and numerous migrated leukocytes. Diphtheritic inflammations tend to form pseudomembranes, which are grayish white, yellowish white, dirty gray, or if mixed with a great number of erythrocytes, dark gray or dirty brown in color. These membranes, when removed artificially, or when shed in the natural course of the necrosis, leave a raw, ulcerated, often bleeding surface.

BACILLUS DIPHTHERIÆ.

Occurrence and Pathogenesis.—Diphtheritic inflammations occur in man and the domestic animals. In man diphtheria is generally a disease of the tonsils, pharynx, and larynx, although it may also occur in accidental or operative wounds. It is caused by the bacillus of diphtheria.

Morphology.—This bacillus, as found in recent diphtheritic inflammations or obtained from pure cultures raised on Loeffler's blood-serum mixture, shows the following morphologic features: The bacillus varies considerably in length from 1 to 6 micra; the majority being about 3 micra. It is from 0.3 to 0.8 of a micron thick. In shape it is frequently slightly curved, and in very rare cases cylindrical. It is generally thickened at one end or more rarely at both ends, making it either club-shaped or dumb-bell-shaped. When it forms chains they are always short. On division and multiplication the bacilli have a tendency to separate immediately at the

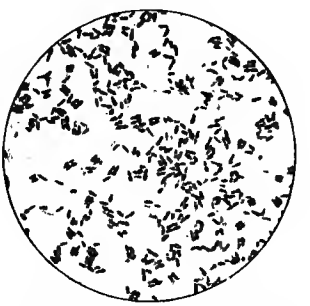
constricting line of division and are, for this reason, frequently found in groups of parallel rows. These groups somewhat resemble pallisades, and, hence, a *pallisade arrangement* of the diphtheria bacilli from pure cultures is spoken of. The bacillus does not form spores and is not motile.

Cultural and Staining Properties.—The diphtheria bacillus or Klebs-Loeffler[1] bacillus is best stained with Loeffler's *alkaline methylene blue.* When derived from a young blood-serum culture (eighteen to twenty-fours hours' incubation) it generally shows very typical staining properties. It does not take the dye uniformly, so that stained spaces alternate with unstained spaces. The stain is generally taken at either end and by a segment in the middle. This causes diphtheria bacilli frequently to appear to the beginner like short chains of streptococci, but a more careful examination will show them to be unequally

FIG. 116

One of the very characteristic forms of diphtheria bacilli from blood-serum cultures, showing clubbed ends and irregular stain. × 1100 diameters. Stain, methylene blue. (Park.)

FIG. 117



Pseudodiphtheria bacilli. (Park.)

stained bacilli. When grown on *agar* they do not show this typical behavior, but become much shorter and stain more uniformly, resembling then more closely the non-pathogenic bacillus known as the *pseudodiphtheria bacillus.* On this account Loeffler's blood-serum mixture[2] should always be used in making cultural inoculations from suspected diphtheria cases. The Bacillus diphtheriæ retains Gram's stain. It also grows on *agar,* on *bouillon,* and in *milk.* A twenty-four hour culture on *blood-serum mixture* or *agar* shows comparatively small, grayish-white, granular, moderately dry or slightly moist colonies. The bacillus grows in the presence or absence of oxygen, best at blood temperature. It does *not liquefy gelatin* and does not grow well on it. It forms gas in the presence of glucose. Pure cultures are generally obtained by first inoculating the blood-serum mixture,

[1] Named Klebs-Loeffler, after its discoverers.

[2] The formula for Loeffler's blood-serum mixture is given on p. 133.

keeping it in the incubator for eighteen hours and then pouring plates with *glycerin agar*. In the cultivation of larger masses of the bacilli for the production of the toxin, bouillon is generally employed. Some strains of bacilli grow readily and abundantly on it, others only feebly. The *bouillon* should be slightly alkaline to litmus. Frequently the growing and multiplying bacilli, after twenty-four to forty-eight hours, produce a diffuse cloudiness in the bouillon and form a film or pellicle on its surface.

Toxin Formation.—The diphtheria bacillus forms a soluble, very poisonous toxin, which when inoculated into a susceptible animal, produces all the general symptoms of the disease.

Diphtheria Antitoxin.—This is prepared by the systematic injection of diphtheria toxin and subsequently cultures of diphtheria bacilli into a perfectly healthy horse. The technique and details are almost identical with those employed in the preparation of tetanus antitoxin, and are fully described in the chapter on the Bacillus tetani.

Animals Susceptible.—Young cats in houses where diphtheria occurs among children, frequently contract the disease. Guinea-pigs, young cats, young rabbits, and other animals can easily be infected experimentally. Many animals are susceptible to experimental intraperitoneal injection with diphtheria toxin.

In making a diagnosis of diphtheria the possibility of the presence of the *pseudodiphtheria bacillus* must always be considered. As the latter cannot always be distinguished morphologically from the true Bacillus diphtheriæ, animal experiments are sometimes necessary to decide the question.

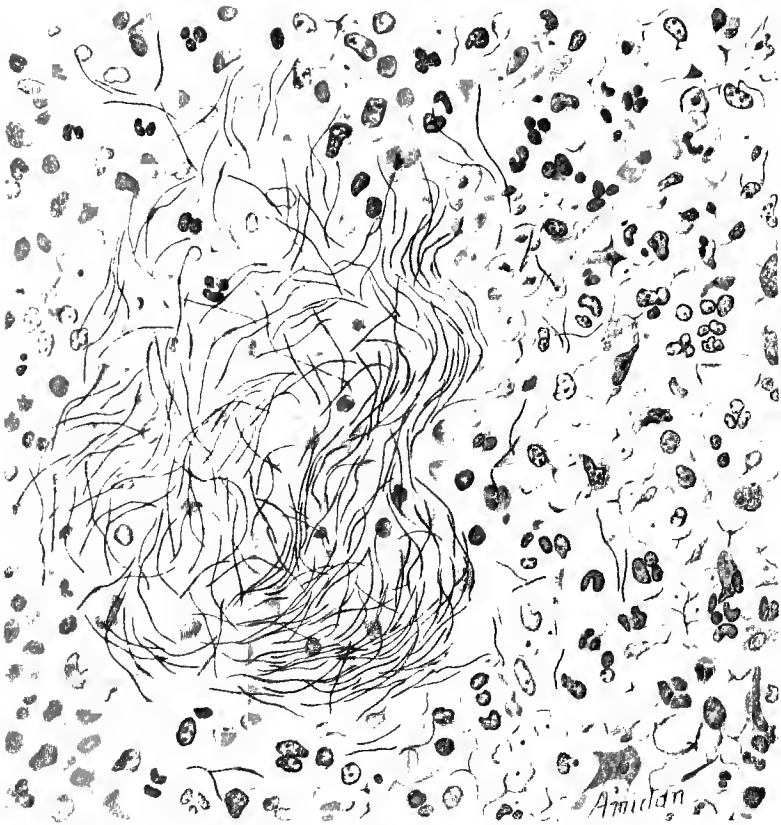
BACILLUS NECROPHORUS.

The Bacillus necrophorus was first found by Loeffler in diphtheria of calves and called by him Bacillus diphtheriæ vitulorum (Latin, *vitulus*, a calf). It is also known as Streptothrix necrophora, Bacillus necroseus, Streptothrix cuniculi (Latin, *cuniculus*, a rabbit).

Occurrence and Pathogenesis.—It is a very common cause of diphtheritic, necrotic inflammations among domestic animals. Ostertag, quoting Bang, enumerates the following pathologic conditions in which it has been found:

Diphtheria of calves.
Furunculosis of cattle.
Dry gangrene of the udder of cows.
Multiple necrotic foci in the liver of cattle.
Multiple abscess in the liver of cattle.
Diphtheritis of the uterus and vagina of cows.
Diphtheritic necrosis of the small intestines of calves.
Embolic pulmonary necrosis in cattle.
Embolic myocardial necrosis in cattle.

PLATE IV



Section of the Lung of a Horse. Bacillus Necrophorus Infection.

Wound necrosis in cattle.
Necrosis of the hoof cartilages of the horse.
Diphtheria of the intestines in horses.
Diphtheritic necrosis in the mouth, nose, and intestines of hogs.
Multiple necrosis in the liver of sheep.
Multiple necrosis in the liver of mules.

Diphtheria of Calves.—In the diphtheria of calves the mouth and pharynx show diphtheritic pseudomembranes. In advanced and fatal cases diphtheritic necrosis is also found in the intestines and lungs. The affected areas appear as yellowish, irregular, necrotic patches, covered by diphtheritic membranes.

Multiple Liver Abscesses in Cattle.—Multiple necrotic foci or multiple abscesses, sometimes as large as an apple and even larger, are frequently found in the livers of cattle. They are surrounded by a tough, fibrous connective-tissue capsule and contain a very tenacious, thick, generally greenish non-fetid pus, in which there is much granular necrotic material. In it bacilli and filaments are found which by culture and inoculation experiments can be identified as the Bacillus necrophorus. Occasionally the bacillus is associated with the Bacillus pyogenes bovis.

Furunculosis of the Horse's Hoof with Necrosis of the Cartilages.—Necrophorus infection of the hoof of the horse is very common in certain seasons and localities. It appears particularly in winter and was very prevalent in 1909 and 1910. The infection causes progressive necrosis of the cartilages and often leads to changes which permanently damage the locomotion of the animal. In spite of antiseptic treatment and operative procedures the process tends to spread. Sometimes it also produces a general infection with the formation of multiple metastatic bacterial emboli in the liver and lungs. The author has seen the case of a horse which died of a general necrophorus septicopyemia with the formation of metastases in the internal organs. The lung presented the picture of a lobular or bronchopneumonia and in the consolidated areas the Bacillus necrophorus was found in enormous numbers.

Foot-rot and Lip-and-leg Disease of Sheep.—Mohler and Washburn have fully established the Bacillus necrophorus as the etiologic factor of this disease, which is now widespread in the United States and of considerable importance. They describe the symptoms, lesions, and course as follows:

"The first evidence of an attack of foot-rot to attract the attention is a slight lameness, which rapidly becomes more marked. Previous to this, however, there has appeared a moist area just above the horny part of the cleft of the foot, and this has gradually reddened and assumed a feverish, inflamed appearance. It may first become visible either at the front or back part of the cleft, but usually the erosions make their first appearance at the heel. The inflammation rapidly penetrates beneath the horny tissue, while from the ulcerous

opening there exudes a thin, purulent fluid. The lameness increases and the region of the foot above the hoof becomes swollen and warm to the touch. The exudates from the erosions contain pus cells, bits of destroyed tissues of the foot, and bacteria. It possesses an odor pungent and disagreeable, but at the same time very characteristic This odor is so pathognomonic of the disease that it would reveal the presence of infected sheep to one familiar with the character of the infection, even before noticing the animals.

"The invasion of the necrotic process may continue until ligaments, tendons, and even the bones are attacked, but before this final stage is reached nature will attempt to repair the damage.

FIG. 118

Leg-and-lip disease in sheep. Infection with Bacillus necrophorus. (Dolan.)

"The hoof of a sheep suffering from a chronic case of foot-rot grows out rapidly and becomes very hard. It will often be found with the toes so thickened and lengthened that the front part of the foot is raised above its natural incline and the tendons at the heel are subjected to additional strain, all of which tends to increase the lameness and the awkwardness in gait of the victim. These thickened and elongated toes will frequently be seen to have attained an added length of three or even four inches, and they curl up like sled runners, greatly interfering with the progression of the animal.

"The course of this disease is slow and protracted, usually starting with one foot and subsequently involving one or more of the others. During this interval it will probably spread to the feet of other sheep,

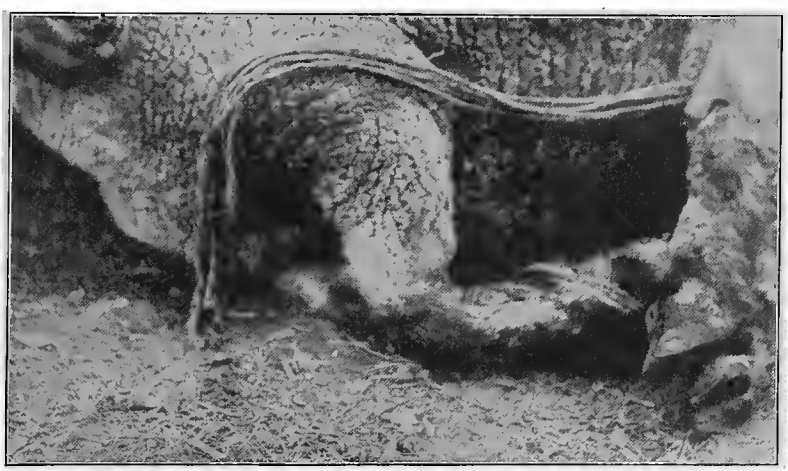
and in this way the disease may remain for several months in each member of the flock, and for eight or ten months in the flock itself.

FIG. 119

Leg-and-lip disease in sheep. Infection with Bacillus necrophorus. (Dolan.)

When the ulcerous processes have become advanced and aggravated, fever develops, the appetite is lost, and the animal grows so emaciated that death intervenes. In some cases that are left untreated recovery

FIG. 120



Leg-and-lip disease in sheep. Infection with Bacillus necrophorus. (Dolan.)

may follow slowly, but there is usually either a dense fungoid growth between the claws, a stiffening of the joints of the ankle, or a long

fissured and misshapen hoof. When treatment is properly applied in the early stages of the disease it is usually cured within ten days. It is very rare for death to occur as a result of foot-rot, although in very virulent outbreaks involving three or four feet of each sheep the affection may terminate fatally within two or three months."

Secondary necrophorus ulcerations frequently occur on the lips of sheep as a result of infection from licking the ulcerations on the feet. The disease is then termed lip-and-leg ulceration. The contagion may also affect the male and female genitalia, in which case it is known as necrotic venereal disease of sheep.

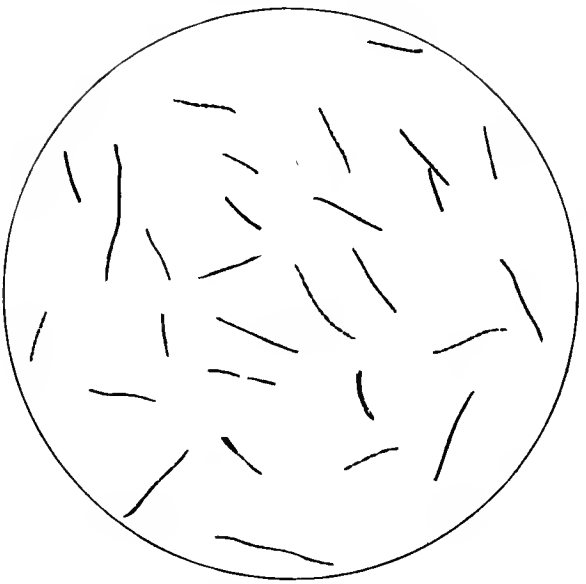
FIG. 121

Leg-and-lip disease in sheep. Infection with Bacillus necrophorus. (Dolan.)

Morphology and Staining Properties.—The organism, when found in pus and necrotic material, appears not only as a bacillus, but also in long filaments, which give it the character of a streptothrix. The filament sometimes break up into very short segments, and may then appear like a true streptothrix. The filaments are from 80 to 100 micra in length. The organism is best stained with Loeffler's methylene blue, carbol fuchsin, or carbol thionin. In stained specimens of the filamentous type, unstained spaces often alternate with short, stained, cylindrical rods, causing the filament to look somewhat like a stepladder. In sections of tissues the bacilli, threads, and filaments show a radial arrangement, and they are seen most clearly and in greatest numbers at the boundary zone between the necrotic and the hyperemic tissues. Branched forms frequently occur. It does not form spores and the long filaments are not motile, but the short bacilli when first seen in pus, exhibit a slight motion. Flagella, however, have not been demonstrated, and the motility is soon lost.

Cultural and Biologic Properties.—The organism is strictly anaërobic and will not grow in the presence of oxygen, It grows best on blood serum or a mixture of agar and blood serum at 30° to 40° C. It also develops on agar and gelatin. In *stab cultures in high blood-serum tubes* small whitish points appear along the stab after twenty-four to forty-eight hours. When a maximum of growth has been reached, which takes six or eight days, an opaque, grayish-white, cylindrical mass, surrounded by a transparent zone of small individual colonies appears along the stab. Its great susceptibility to oxygen and its

PLATE V



Cover-glass Preparation from Pure Culture of Bacillus Necrophorus. (Mohler and Washburn).

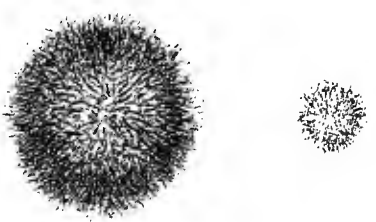
FIG. 122

Bouillon-agar culture (first dilution) of Bacillus necrophorus, showing twenty-four-hour growth, with numerous small gas-bubbles, but the colonies have not developed sufficiently to become visible. (Mohler and Washburn.)

FIG. 123

Seven-day-old bouillon-agar culture of this organism of the fourth dilution. The isolated colonies are characteristic in that their grayish centres are surrounded by fuzzy white areas, not unlike the strands of loose, fleecy cotton. (Mohler and Washburn.)

FIG. 124



Single colonies of the necrosis bacillus, showing filamentous character of the growth (enlarged about seven diameters). (Mohler and Washburn.)

strictly anaërobic properties are shown by the fact that the culture never reaches the surface of the serum medium. In the condensed water of *serum agar* the growth forms a grayish-white film or pellicle and a sediment of the same color. When grown in *bouillon* or *milk* a smell of cheese is given off; the fluid media then give the *indol reaction.* The organism is frequently found in the intestines of herbivorous animals, particularly the hog. Pure cultures cannot, as a rule, be obtained directly from the lesions, because the organism is rarely present in that condition, but is usually mixed with other pathogenic or saprophytic organisms. The method of procedure consists in inoculating the material subcutaneously into a rabbit. A very hard inflammatory induration is then formed at the site of the injection, where the tissues subsequently became caseous and necrotic. The necrophorus bacillus at first multiplies locally, and afterward enters the general lymph and blood circulation, leading to the formation of metastases in the internal organs, particularly the lungs and the liver. The rabbit generally dies in less than two weeks, and anaërobic cultures can be made from the internal metastases, which usually contain the organism in pure culture. The disease also occurs spontaneously in rabbits, starting as an infection of the face.

BACILLUS DIPHTHERIÆ AVIUM.

Occurrence and Pathogenesis.—A highly contagious disease characterized anatomically by diphtheritic inflammations of the mucous membranes of the head occurs among pigeons, domestic and prairie chickens, turkeys, and other fowl. The diphtheritic pseudomembranes are found in the mouth, pharynx and nose, and its accessory cavities, less frequently in the larynx, trachea, bronchi, and intestines. In the latter the cecal pouches are sometimes completely filled with the diphtheritic membranes. The cause of the disease is a short bacillus which was first found by Loeffler in diphtheria of pigeons. It was, accordingly, called Bacillus diphtheriæ columbarum. Later, Moore and others found the same bacillus in chicken diphtheria, and it is now generally known under the more general term of the bacillus of bird diphtheria (Bacillus diphtheriæ avium).

Morphology and Staining Properties.—The organism is a short bacillus, not motile, and does not form spores. It stains in a bipolar manner with the watery anilin stains, and is Gram negative.

Cultural Properties.—It grows both in the presence and absence of oxygen. On *gelatin,* which is not liquefied, a grayish-white transparent growth composed of finely granular colonies appears first, later the growth becomes white and opaque. On *agar* the growth is similar, but first slightly bluish, then white and opaque, as on gelatin. *Bouillon* becomes first uniformly cloudy, later a somewhat transparent white

sediment is formed. On *potatoes* either a yellowish-white or a grayish-white growth is formed, but occasionally the organism refuses to develop on this culture soil. The organism is also pathogenic for mice and rabbits.

QUESTIONS.

1. What are the characteristic pathologic changes in diphtheritic inflammations?
2. What is the cause of diphtheria in man?
3. Describe the organism causing the disease.
4. Describe its staining properties when stained with Loeffler's alkaline methylene blue. Give the formula for this stain.
5. Give the formula for the preparation of Loeffler's blood-serum mixture.
6. Describe the growth of the Bacillus diphtheriæ on this culture medium.
7. What non-pathogenic bacillus in cultures and stained microscopic specimens looks very much like the Bacillus diphtheriæ?
8. How does this bacillus act toward oxygen, glucose, gelatin?
9. What kind of toxins are formed by the diphtheria bacillus?
10. What domestic animals contract diphtheria spontaneously?
11. Name a number of animal diseases due to the Bacillus necrophorus.
12. What other names have been given to the Bacillus necrophorus?
13. What is the arrangement of this bacillus in infected tissues?
14. What are the cultural and biologic properties of this organism?
15. How are pure cultures obtained from pathologic material containing the Bacillus necrophorus and other bacteria?
16. Describe the pathologic lesions caused by the organism in calves.
17. Give the description and common cause of multiple liver abscesses in cattle.
18. What affection of the horse's hoof is frequently caused by the Bacillus necrophorus? What occurs if this hoof disease leads to the death of the animal?
19. What disease of sheep is caused by the Bacillus necrophorus?
20. Describe the pathologic lesions as seen in the feet of affected sheep.
21. What other parts of the body of sheep may become infected with the Bacillus necrophorus?
22. What animals are spontaneously infected by the Bacillus diphtheriæ avium?
23. Describe the pathologic lesions produced.
24. Give the morphologic and cultural properties of the Bacillus diphtheriæ avium.

CHAPTER XIX.

BACILLI OF THE HEMORRHAGIC SEPTICEMIA GROUP—BACILLUS AVISEPTICUS, BOVISEPTICUS, OVISEPTICUS, SUISEPTICUS, AND EQUISEPTICUS—BACILLUS OF DOG TYPHOID—PLAGUE BACILLUS IN MAN AND ANIMALS.

MANY bacterial infections, when they take a violent, rapidly fatal course, may lead to hemorrhagic septicemia, that is, to a general infection of the blood with extensive multiplication of the bacteria in the blood current and with the formation of areas of hemorrhagic inflammation in the mucous and serous membranes and in the various internal organs of the body. A staphylococcus or a streptococcus infection may lead to a hemorrhagic septicemia of this kind, but considering the great number of infections with these organisms in man and domestic animals this result is not very common.

On the other hand, certain bacteria, in a great majority of cases, lead to such hemorrhagic septicemias, and they are known as *the group of bacilli of hemorrhagic septicemia.* These organisms have a number of common features. All are rather small, short bacilli, with rounded ends, which do not form spores, do not liquefy gelatin, are non-motile and Gram negative, and stain in a peripheral or polar manner so that on first sight they often appear like diplococci. Other bacteria, such as the anthrax bacillus, also commonly produce a hemorrhagic septicemia, but they have different morphologic, cultural, and biologic properties, and do not belong to this group.

Historical.—Rivolto and Semmer, in 1878, and Pasteur, in 1880, described a bacillus of the type indicated as the cause of fowl cholera; Gaffky, in 1881, a similar one as the cause of septicemia in rabbits; Kitt, in 1883, one for the disease among wild animals called "Wildseuche," by Bollinger; and Loeffler, and later Smith, in 1886, one as the cause of swine plague or "Schweineseuche." Hueppe, from his studies of the various bacilli of this group, concluded that they were more or less identical, and proposed to classify them as the group of bacilli of hemorrhagic septicemia. It was subsequently ascertained that bacilli of this group are also the cause of *infectious pleuropneumonia of calves, barbone disease of buffaloes,* the *hemorrhagic septicemias of cattle, infectious pneumonia of goats, infectious pneumonia of horses,* and a *hemorrhagic gastro-enteritis of dogs.* Lignière and Trevisans, in 1890, gave the name of Pasteurella to the diseases of this group, and designated the bacteria themselves as *Pasteurelloses.*[1]

[1] Kitt, with all due respect for the genius of Pasteur, severely criticized an attempt of this kind to change well-known names, which could only lead to confusion in the nomenclature of diseases and their causative factors. He ironically remarks that a general adoption of such a principle would lead to names of diseases and their bacteria like Kochella (for tuberculosis), Loefflerella or Schuetzella (for glanders), Schulzerella, Millerelose, Smithellose, etc.

These names, however, have not been generally adopted and are only used by the French. It is preferable, as Hutyra and Marek point out, to retain the name *Bacillus bipolaris septicus* and to use as distinguishing designation the names *Bacillus avisepticus, bovisepticus, suisepticus,* etc. Kitt proposed the term *Bacillus plurisepticus* as the common name for all these bacteria. The disease which they produce in such a large variety of animals he designated as *septicæmia pluriformis* or *septicæmia polymorpha.*

BACILLUS AVISEPTICUS (BACILLUS OF FOWL CHOLERA).

Historical and Occurrence.—This disease of chickens and other domestic birds is also known as *chicken cholera, fowl typhoid* (Geflügeltyphoid, German), *Pasteurelosis avium* (cholera des poules, French). It is one of the diseases due to bacilli of the hemorrhagic septicemia group, and has long attracted the attention of breeders and veterinarians. It was first described toward the close of the eighteenth century, and its extremely contagious character was recognized by Delafond and Renault in 1851. Perroncito, in 1878, first saw the causative microörganisms in the blood of fowls which had died from the disease, but he mistook it for a diplococcus, an excusable error at this early stage of the study of pathogenic bacteria. Toussaint, in 1879, and Pasteur, in 1880, confirmed Perroncito's findings. The two French investigators also cultivated the organism in chicken broth, and Pasteur showed that it could be attenuated in artificial cultures and afterward used as a protective virus. This work of Pasteur was the first of its kind, and it became a fruitful source of further attempts in the preparation of attenuated cultures to be used as protective vaccines. Kitt, Lignière, Rivolta, Zuern, Celli, Solomon, and others subsequently studied the bacillus causing fowl cholera, and with the exception of the bacillus of bubonic plague, it is at present the best-known organism of the group of bacilli of hemorrhagic septicemia.

Fowl cholera is very prevalent in Europe (with the exception of Great Britain) and South Africa, and it has been found also in the United States and Canada. According to official statistics, 48,797 chickens, 23,573 geese, and 9488 ducks died from fowl cholera in Germany in 1903. These figures comprise only those cases in which an exact diagnosis was made, and indicate, of course, only a part of the actual loss.

Pathologic Lesions.—Birds which have died from fowl cholera show numerous hemorrhages into the mucous and serous membranes. Among the serous membranes the epicardium, or external lining membrane of the heart, shows particularly numerous hemorrhagic spots. This change is most marked in geese and ducks, less in chickens. A serous or fibrinous pericarditis, a hemorrhagic enteritis

(inflammation of the intestines), and sometimes an acute serous pneumonia is also present. According to Ward the most striking intestinal lesions are found deeply in the first and second duodenal flexures. The mucosa is deeply reddened and studded with hemorrhages, varying in size, but seldom exceeding one millimeter in diameter. These hemorrhages involve the intestinal coats to such an extent that they are distinctly visible on the peritoneal surface. The contents of the duodenum consist of a pasty mass more or less thickly intermingled with blood clots. Marked lesions are very rarely observed in other portions of the intestines. In cases where these morbid changes are not so well marked, diagnosis with the naked eye is impossible, and the blood must be examined microscopically and other birds inoculated.

Morphology and Staining Properties.—The organism causing fowl cholera is now generally known as the *Bacillus avisepticus* (Latin, *avisepticus*, septic for birds). It is one of the smallest of the group and is rarely longer than one micron. The bacilli are frequently seen in the blood as round or oval bodies; they stain with the ordinary watery anilin stains in a polar manner, and can be easily mistaken for diplococci. They are Gram negative, immobile, possess no flagella, and form no spores. Enormous numbers are present in the blood of sick and dead birds. The diagnosis from the blood must be made either before or shortly after death, because putrefaction bacteria closely resembling the Bacillus avisepticus frequently develop in cadavers of birds.

Cultural Properties.—In a *gelatin stick culture* made from the blood of a bird and kept at room temperature, densely crowded, small, translucent whitish points appear after a few days along the line in the gelatin. These, later, become confluent and form a white filiform mass. On the surface, small, delicate, transparent dewdrop-like colonies appear, and later become more decidedly white. The gelatin does not become liquefied. On *agar slants* the development is similar but quite scanty, and the colonies generally do not become confluent but remain small. The growth on *blood serum* is generally more abundant, and leads to a thin, dull white film over the entire surface. The organism generally does not grow on potatoes. The growth in *nutrient bouillon* is abundant. It produces clouding of the medium, a sediment at the bottom of the tube, and sometimes a thin, delicate pellicle on the surface.

Susceptible Animals.—If chickens, geese, ducks, pigeons, turkeys, sparrows, etc., are inoculated subcutaneously with a minimum amount from cultures of the Bacillus avisepticus, they develop a rapidly fatal hemorrhagic septicemia. Rabbits and mice are also quite susceptible, and succumb to the infection. Cattle, sheep, and horses, after subcutaneous infection, develop local abscesses containing numerous bacteria, but there is no general infection. Guinea-pigs, after subcutaneous inoculation, develop a local abscess only, but after an intraperitoneal application die from a septic peritonitis. Repeated

passage of the bacteria through the bodies of susceptible animals increases its virulency, and exposure to air and light lessens it.

Natural Infection.—Among fowl, infection is, as a rule, transmitted through the gastro-intestinal tract. It has been ascertained that fowls contract the disease from eating infected organs and food soiled with feces from infected birds. The bacteria, after ingestion, invade first the lacteals, then the lymph clefts, and from them the blood current. It is also highly probable that ectogenous parasites (lice) can spread the disease from sick to healthy birds.

Resistance.—In moist soil protected against air and light the Bacillus avisepticus can remain alive and virulent for a considerable time; in manure, according to Gärtner, at least one month; in putrefying cadavers, according to Kitt, three months. It is not killed by freezing, but it soon perishes when dried out. It loses its virulency, according to Kitt, when exposed in the moist condition for one-half hour to 45° to 46° C.; it is killed at from 80° to 90° C. in five to ten minutes. Solomon and others have studied the effects of disinfectants upon the bacillus, and have found that chlorinated lime in a dilution of 1 to 100; slacked lime, 1 to 20; sulphuric acid, 1 to 300; hydrochloric acid, 1 to 500, and 1 per cent. carbolic acid rapidly kill the organism. Repeated whitewashing of infected places is an excellent means of disinfection, but before application the woodwork should be washed with hot soda solution and the floors scrubbed with creolin or lysol solution. Sick animals must be separated from the healthy, and cadavers should be deeply buried, or, better still, burned. According to the observation of several investigators the bacilli are frequently found as saprophytes in the outside world.

Immunization.—The first experiments in protective inoculation against this bacillus by attenuated cultures were made by Pasteur. Attenuated cultures powerful enough to kill pigeons will only cause local necrotic processes in chickens, geese, and ducks. *Passive immunization* has been practised (Kitt) with a serum from horses hyperimmunized against the Bacillus avisepticus. Jensen has observed that chickens which have passed through an infection with the bacillus of the hemorrhagic septicemia of calves were subsequently immune against infection with the Bacillus avisepticus. The latter, it has been claimed, produces a soluble toxin which will pass a Pasteur filter. Since it is known, however, that very small bacilli sometimes pass through a Berkefeld filter, it is possible that the transitory effects of the filtrate depended upon the presence of a very few not very virulent bacilli.

Other Septicemias among Birds.—In addition to the common fowl cholera a number of other septicemias in birds have been described. They are evidently not identical with the avisepticus infection, and are caused by different organisms. Some of these affections are:

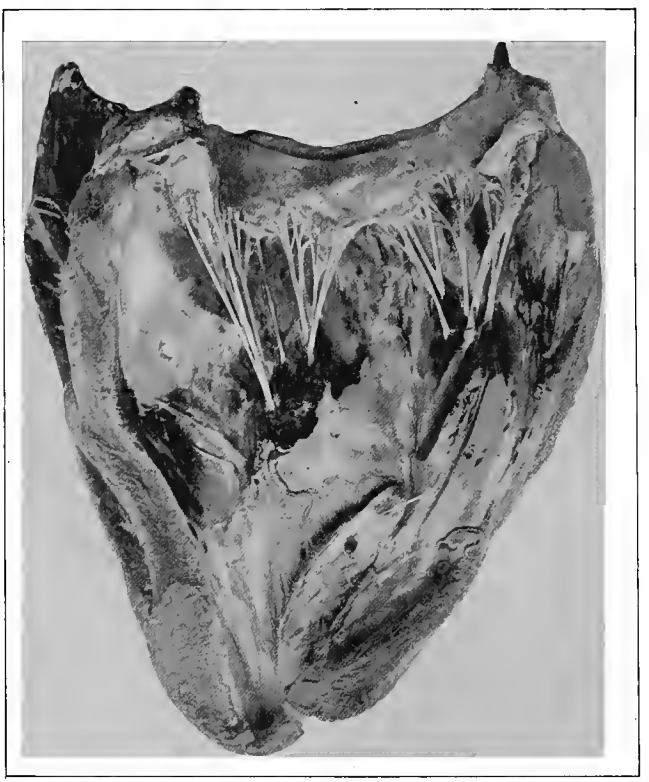
A disease observed among pigeons in New Jersey by Moore; one observed among chickens by Noergaard and Mohler, caused by a

streptococcus also pathogenic to rabbits, mice, pigeons, and dogs; and another among domestic birds caused by the spirillum of Metchnikoff (see under spirilla). Another contagious disease of chickens with the characteristics of a septicemia has been observed in Lombardy, Austria, and Germany. It is transferable through the blood of sick animals, and is due to an invisible, filterable, contagious virus.

BACILLUS BOVISEPTICUS (BACILLUS OF HEMORRHAGIC SEPTICEMIA OF BOVINES).

Occurrence.—Bollinger, in 1878, first described a disease occurring in Bavaria among wild deer and wild hogs, which later spread to

FIG. 125



Interior of right ventricle of the heart of a cow showing hemorrhagic endocarditis as it occurs in hemorrhagic septicemia. (Reynolds.)

domestic cattle and hogs, and even horses. While he recognized its infectious character, Kitt, in 1885, was the first to discover its cause,

and Hüppe, in 1886, to study it in detail. The disease has since been found in various parts of Germany, Austria, and other countries of Europe, the United States, Indo-China, the Malayan Peninsula, Java, Hongkong, the Philippine Islands, and also in Algiers, in Africa.

Pathologic Lesions.—The pathologic lesions are those of a hemorrhagic septicemia; namely, general congestion, petechial hemorrhages, and ecchymoses into the mucous and serous membranes and internal organs. The liver and kidneys are swollen and cloudy, but the spleen, unlike its condition in anthrax with which this hemorrhagic septicemia may be confounded, is not enlarged, and has either a general normal appearance or contains multiple, circumscribed hemorrhages. In the so-called exanthematous or edematous form the subcutaneous connective tissue of the head and neck contains a watery and partly hemorrhagic infiltration. The tongue is frequently more or less enlarged, dark or dirty brown red, and of a firm consistency. The mucous membranes of the mouth and upper respiratory tract and the retropharyngeal and cervical glands are swollen. The mucosa of the intestinal tract is also swollen and hemorrhagic. In some cases the lungs show not merely congestion, but areas of consolidation, and the pericardium, in addition to the pericardial hemorrhages, may show the signs of a fibrinous inflammation. Reynolds reported an outbreak of the disease in Minnesota in which meningeal lesions of the brain and cord were very marked. The disease is caused by the Bacillus pluriformis of Kitt, now more generally known as the Bacillus bovisepticus.

Morphology and Staining Properties.—The bacillus possesses the same morphologic and staining properties as the Bacillus avisepticus. It is from 0.6 to 1 micron long, sometimes a little larger, and 0.3 micron wide. It is found in considerable numbers in the blood of animals in the last stages of the disease or in those dead from it, and in enormous numbers in the blood of artificially infected rabbits or mice, which are exceedingly susceptible. It often appears in the blood in short chains. In doubtful cases the diagnosis should not be made until rabbits or mice have been inoculated. These die in from twelve to twenty-four hours.

Cultural Properties.—Wilson and Brimhall describe the cultural properties as follows: The organism is aërobic, but prefers the depth rather than the surface of the medium. It grows best at the body temperature and more slowly at room temperature. In ordinary *nutrient* and in *glucose bouillon* a heavy growth appears in twenty-four hours. In *Dunham's solution* a small amount of indol is formed in forty-eight hours. *Milk* is not coagulated. In *gelatin plates* small, white, granular colonies appear after forty-eight hours. In *gelatin stab cultures* a light growth occurs on the surface, while along the needle track numerous colonies like those in the deep portions of the plate develop in the culture. The organism grows on neutral but not on acid *potatoes*.

Resistance.—The organisms are destroyed in fluids at 58° C. in seven or eight minutes; by corrosive sublimate solution 1 to 5000 in one minute.

Natural Infection.—The common mode in cattle seems to be through the intestinal tract. It appears that the Bacillus bovisepticus is widespread as a saprophyte in nature, and that it may lose most of its virulency, but also regain it under conditions which are not yet well known.

Other Septicemias among Bovines.—*Corn-fodder Disease.*—It was formerly believed that the so-called corn-fodder or corn-stalk disease, which sometimes occurs extensively among cattle along the upper and middle Mississippi Valley, was due to the Bacillus bovisepticus, but according to Moore's investigations this certainly does not appear to be the case.

Septic Pleuropneumonia of Calves.—This disease probably differs only clinically from the typical hemorrhagic septicemia of cattle, and is due to the Bacillus bovisepticus, though it has been claimed that it is caused by a somewhat different variety.

Hemorrhagic Septicemia of Sheep.—This disease has been observed in Europe and Argentina. It presents the typical pathologic changes seen in the other hemorrhagic septicemias due to the bacilli of this group. It is caused by the variety known as Bacillus (bipolaris) ovisepticus.

BACILLUS SUISEPTICUS.

Hemorrhagic septicemia of swine, caused by the Bacillus suisepticus, is commonly known as *swine plague.* There was considerable confusion in the past as to the exact etiology and pathology of this disease, because it is frequently associated with another disease of swine known as hog cholera, and, further, because both diseases may occur as a mixed infection in a single animal.

The nomenclature is another unfortunate and confusing feature. The English word "swine plague" literally translated into German is "Schweinepest," just as the English term bubonic plague is "Beulenpest." The German word "Schweinepest," however, is the name for the disease called hog cholera in English and not for swine plague. This explains why so much confusion has arisen in the discussion of these two diseases of swine. Swine plague is due to a bacillus of the hemorrhagic septicemia group, while hog cholera, formerly believed to be due to a bacillus of the typhoid colon group (the hog cholera bacillus), is most probably due to an invisible, filterable virus.,

Historical and Occurrence.—Loeffler, in 1886, discovered that the septicemic form of swine plague was due to a specific organism which was later classified with the other bacilli of the bipolar group. Schütz, in the same year, demonstrated the presence of the identical bacillus in the pectoral form of the affection. The later contributions by

Salmon, Smith, Moore, Jensen, and Bang confirmed the early work of Loeffler and Schütz. The disease has been found in several European countries, and is quite widely spread throughout the United States.

Pathologic Lesions.—In the most acute cases the changes found in the dead animals are those of a typical hemorrhagic septicemia, with petechial and ecchymotic hemorrhages into the skin, subcutaneous fat, and the serous and mucous membranes. The kidneys, including the subscapular tissue, the pelves, and even the parenchyma, very frequently are the seat of blood extravasation. The membranes of the brain are likewise frequently hemorrhagic; the lymph glands show a condition of acute hemorrhagic inflammation. In cases which have not taken the most acute course the lungs are generally found involved. They show multiple areas of consolidation which are at first dark brown red and later a lighter grayish red. The foci of consolidation contain necrotic material, and in older cases the entire lobe of a lung may have become changed into a mass of caseous necrotic substance. The non-consolidated pulmonary tissue is edematous, the pleura thickened, congested, and covered by a fibrinous exudate. The pericardium may show similar changes, and the pleural cavity may contain a varying amount of hemorrhagic seropurulent fluid. The mucosa of the gastro-intestinal tract is swollen and hemorrhagic and sometimes covered by rather thin pseudomembranes, formed by superficial necrotic cells. The spleen is not enlarged, the kidneys are congested. According to Moore the pneumonia of swine plague presents itself both as a lobar and as a lobular bronchopneumonia. In the former the alveoli become filled with red and white blood corpuscles and fibrin. In chronic cases the animals are much emaciated, and the lungs, peribronchial and mesenteric glands and tonsils contain necrotic foci.

Morphology and Staining Properties.—In the most acute cases the Bacillus suisepticus (also called Bacterium suicidum) is found in great numbers in the blood and internal organs. In the less acute cases it is found in the consolidated and necrotic foci in the lungs, occasionally also in the blood. In the chronic cases the organisms do not occur in the blood, but as necrotic foci in combination with other bacteria, such as streptococci, staphylococci, Bacillus pyogenes suis, Bacillus necrophorus, and others. The bacillus suisepticus is one micron to a micron and a fraction long, 0.5 to 0.6 wide. It is sometimes almost oval or round; at times it stains quite uniformly, at other times in a bipolar manner. Longer bacilli occasionally stain like the diphtheria bacillus, at both ends and in the centre. The bacillus stains with the ordinary anilin stains, is Gram negative, not motile, possesses no flagella, but a capsule can be demonstrated by proper staining methods. The organism, like that of bubonic plague in older lesions, is often of a swollen vacuolated type, stains only with a peripheral ring, and then has the appearance of an empty shell.

Bang, therefore, called it the *vacuole bacillus*. Loeffler has described a Bacillus parvus ovalus which was probably a Bacillus suisepticus of this vacuolated type.

Cultural Properties.—The Bacillus suisepticus grows at room temperature in artificial culture media, particularly *gelatin*. It grows well on *coagulated blood serum* in the incubator, not so well on *agar* or in *bouillon*. When the growth on agar is more abundant it forms a sticky, slimy layer. It generally does not develop on *potatoes*. It sometimes forms *indol* and sometimes *phenol*.

Animals Susceptible.—Mice and rabbits are very susceptible. They can be easily infected from a small wound, and die very rapidly after the inoculation. Guinea-pigs, pigeons, and chickens sometimes succumb to the infection, at other times they are not susceptible.

Resistance.—According to Moore and Smith the resistance of the Bacillus suisepticus to physical and chemical agencies is as follows: It is killed if heated in bouillon for ten minutes to 58° C. Complete drying out destroys it in twenty-four to thirty-six hours. It does not live in soil over a week and in water not over eleven days. It is killed in lime water in one minute, in 1 per cent. carbolic acid in five minutes, and in formalin, 1 to 1000, in five minutes. The organism is, accordingly, not very resistant.

Immunization.—The first experiments in the immunization of small laboratory animals and swine against the Bacillus suisepticus by attenuated cultures were made by de Schweinitz and Smith. The latter, in 1891–92, reported successful immunization of rabbits and swine, and, in association with Moore, again in 1894. Ostertag and Wassermann have shown, independently, that animals immunized with a certain stem of Bacillus suisepticus are subsequently immune only against this stem, and not against any other stem. At best they are only immune against a few stems, and never against all that might subsequently be employed. This condition, which was confirmed by others, is not absolutely without a parallel, as it is met with also in certain bacilli of the coli group. Ostertag and Wassermann later prepared a polyvalent antiswine plague serum by injecting a number of horses, each with several stems, and then mixing the sera of the different horses. According to these authors, and also Joest, Raebinger, and others, the use of the polyvalent serum has been very satisfactory in Germany. From other sources the reports have not been so favorable.

BACILLUS EQUISEPTICUS.

Equine influenza, known also under the names of *pink-eye, typhoid fever, epizoötic catarrhal fever, horse distemper, mountain fever*, "Pferdestaube," "Rothlaufseuche," and "Brustseuche" (German); Pasteurelosis equorum, la grippe, fievre typhoid, pneumonia

infectieuse (French), is a disease of horses claimed by a number of observers to be due to the Bacillus (bipolaris) equisepticus.

Occurrence.—The disease occurs sporadically, and also as widespread epidemics. It has been observed in Europe, America, and South Africa. It became prevalent in the eastern part of the United States in 1872–3, and from there has spread over the entire country.

Pathologic Lesions.—In the *catarrhal* form the mucous membranes of the respiratory and gastro-intestinal tract show an intense congestion often accompanied by edematous infiltrations, particularly in the submucous connective tissue of the larynx, the pylorus, and the small intestines. The subcutaneous connective tissues are likewise often infiltrated with a watery exudate. Peyer's patches of the small intestine and the mesenteric glands are swollen, and are reddish gray on section. The pleural and peritoneal cavities frequently contain a reddish, cloudy, serous fluid. The spleen shows some swelling, and the lungs are edematous. In the *pectoral* form the lungs contain lobar or lobular areas of consolidation, and the pleura is in a condition of serofibrinous inflammation. The consolidated areas in the lungs become necrotic, and contain a soft, dirty grayish-brown or greenish, very fetid mass. The necrotic foci sometimes break through the pleura and establish a pneumothorax or pyopneumothorax. In the *catarrhal form* the conjunctivæ of the eyes are intensely reddened[1] (brick red or mahogany red), and the submucous connective tissue is edematous. The eyelids swell, covering the eyeball to a large extent. If the lids are opened forcibly there is an abundant flow of tears, and later a mucopurulent discharge.

Morphology.—The Bacillus equisepticus is described by Nocard and Leclainche, according to Lignière's findings, as follows: It is a slender, short bacillus with rounded ends and of about the same size and staining properties as the bacillus of fowl cholera. In cultures it shows as a very small diplococcus (coccobacillus). It is immobile, strictly aërobic, and Gram negative. It is present in the blood in small numbers only, and difficult to find.

Cultural Properties.—Artificial cultures are difficult to obtain. They require the intraperitoneal injection of 4 or 5 c.c. of the horse's blood, pleuritic fluid or juice from the lungs into a guinea-pig, in which they produce a peritonitis, the exudate from which contains numerous ovoid bacteria, from which culture media are inoculated. *Bouillon* cultures, after twenty-four hours, are uniformly clouded, and the reaction of the medium remains the same even after several days. On *gelatin* at 20° C. very small, round, almost transparent colonies are formed after two or three days. The bacillus does not grow well on plain or glycerin *agar;* it grows in *milk* without coagulating it; no growth occurs on *potatoes.* The best culture medium is *bouillon* to which a small amount of *sterile horse's blood serum* has been added.

[1] The name *pink-eye* is derived from this symptom.

The organism is pathogenic for guinea-pigs, rabbits, mice, dogs, cats, swine, sheep, donkeys, and horses. Whether it is pathogenic for cattle is doubtful.

Natural Infection.—In the horse, natural infection is spread by the secretion from the diseased mucous membranes and lungs and through the feces. These secretions and excretions are particularly infectious while the disease is at its height, but they may also spread the contagium for a long time after the affection has run its course. After infection of a previously healthy animal the bacilli multiply rapidly in the mucous membranes and invade the lymph and blood circulations, producing in very violent rapidly fatal cases the picture of typical hemorrhagic septicemia.

Hutyra has confirmed the observations of Lignière, identifying the Bacillus equisepticus as the cause of horse influenza, or pink-eye, but other authors still consider the etiology unsettled, and doubt whether this organism is the actual cause.

BACILLUS OF DOG TYPHUS.

An acute, epidemic disease of dogs, characterized by a violent gastro-enteritis with ulcerative stomatitis was first described in 1850 by Hofer under the name of *"Hundetyphus,"* dog typhus, and later under the name *gastro-enteritis hemorrhagica*. During the last two decades several larger epidemics have been described in Germany and other parts of Europe. Lignière claims that the disease is due to a bacillus of the hemorrhagic septicemia group. He describes the organisms as having the general characteristics of the group, but that it is larger when it first occurs in the dog and only becomes smaller after having passed through several guinea-pigs. It grows on culture media much like the other members of the group. Lignière's claim has not been confirmed. Hutyra states that he has examined bacteriologically a number of cases, but has not obtained Lignière's bacillus but a bacillus of the *colon group* and another virulent bacillus of the *proteus group*.

BACILLUS PESTIS.

Occurrence and Historical.—The Bacillus pestis, or the bacillus of bubonic plague, is the most extensively studied, and now best-known organism of the group of bacilli of hemorrhagic septicemias. It is the cause of the most dreaded human scourge, *bubonic plague*, the "Great Black Death" of the Middle Ages, which is estimated to have carried away, within a space of three years, twenty-five millions of people in Europe during the fourteenth century. In the last century the disease seemed to have almost if not completely died out, but toward the close a great pandemic broke out in India and China. It spread to various

places in Asia, including the Philippine Islands, to Europe, Africa, Australia, and also invaded the western shores of the United States. The great epidemic in India and China still exists, and is annually killing hundreds of thousands of people. The disease is particularly interesting to the veterinarian, not merely from a general human, but from a special professional standpoint, because it is a natural disease of some rodents, more especially of the various varieties of the common rat. That rats die extensively during plague epidemics had been noticed in the Middle Ages, and certain statements occur in the Bible which evidently refer to rodents dying from plague. The investigations in particular of the English Indian Plague Commission and the Advisory Committee for Plague Investigation in India have demonstrated the great prevalence of acute plague among the rats and its relation to the spread of the disease to man.

Pathologic Lesions of Plague in Rats.—It is of the greatest practical importance to recognize plague in rats. It frequently, if not generally, precedes plague in man, and the human disease cannot be stamped out successfully in a territory unless it is eliminated among the rats. Fortunately, plague in rats can be diagnosticated easily and generally by means of the naked eye without an additional microscopic examination. The plague bacillus, both in rats, other animals, and man, tends to invade the lymph glands nearest to its place of entrance, and there multiplies enormously, leading to very characteristic changes, such as swelling, edema, and hemorrhages into the gland and the neighborhood of the periglandular region. Later the gland may contain pus or a necrotic caseous material. A gland presenting the indicated pathologic changes is called a *bubo*, and, hence, the disease, which so regularly leads to the formation of such buboes, is known as *bubonic plague*. The gland lying closest to the place of infection and first suffering marked pathologic changes is called the *primary bubo* of the first order. If neighboring glands are affected by direct continuous transport of the bacilli they are known as *primary buboes of the second order*. The distant glands which become infected through the blood circulation or through the lymph circulation are known as *secondary, tertiary buboes*, etc. The Advisory Committee for Plague Investigation reported on the examination of over 31,000 rats in Bombay, India, 4000 as infected with plague. The postmortem findings furnished the following general picture. Subcutaneous congestion, visible after removing the skin of the animal, is not infrequently a well-marked feature. It may be general, but in some cases is limited to the bubo. A peculiar purplish-red appearance of the muscles exposed by reflecting the skin of the thorax and abdomen is obviously due to the presence of congested vessels, and combined with the reddish-pink color of the subcutaneous tissue is a strong indication of plague at the beginning of the examination. Subcutaneous hemorrhages are frequently noticed particularly in the submaxillary region; here also edema is common; general edema over the entire body is quite rare.

In a healthy rat the only glands which are large enough to be seen easily are those forming the crescent embracing the salivary glands in the submaxillary region, and the elongated retroperitoneal glands on each side of the middle line in the lower part of the abdomen. The latter are called the pelvic glands. In the bubonic type of plague the glands affected and presenting the picture of the typical plague bubo described above are in the order of frequency of infection, according to the Bombay figures: Glands of the neck (75 per cent.), glands of the axilla (15.1 per cent.), glands of the groin (6.1 per cent.), glands of the pelvis (3.8 per cent.). A bubo in a plague-infected rat feels hard when cut across, but it has not the tough consistency of a normal gland. The contents of the latter are not easily squeezed out by pressure, while in a bubo the substance of the gland is readily broken down by slight pressure with the forceps. A bubo on section has the appearance of necrosis, affecting first the medullary portion of the gland and gradually spreading outward, so that ultimately the gland may be converted into a mass of necrotic tissue enclosed within the capsule. The central portion consequently appears gray, and at a later stage the centre breaks down into a rather dry, very rarely a liquid, purulent material. Buboes with greenish liquid pus are not typical of plague.

The presence of a typical bubo is the most important sign of plague in rats, and next in importance is the so-called granular liver. According to the Bombay findings, it is only met with in rats infected with plague. The liver appears to be in a condition of fatty degeneration, but, as a matter of fact, the changes are not fatty but necrotic. The outlines of the lobules are distinct and the surface of the organ presents numerous gray or whitish granules of the size of a pinpoint, which give the liver a stippled appearance as if it had been dusted over with gray pepper. This appearance has given rise to the term granular liver. The gray areas may be so small that only the closest scrutiny of an experienced observer will detect them. When larger the granules are of a yellow color and vary somewhat in size. In a typical case the granules are not raised above the surface of the liver. The spleen, which is very typically changed in experimental plague in guinea-pigs, is not very characteristically changed in rat plague, though it is generally markedly enlarged. One of the most important postmortem findings in the disease is the presence of an abundant clear pleural effusion. Hemorrhages, both subcutaneous, subperitoneal, and in the internal organs, are likewise constant and important pathologic changes in rat plague.

McCoy has recently published the results of the investigation of rat plague in California, and his findings on comparatively small material, in general, agree well with the Bombay findings. However, in the American material the bubo was generally found in the groin and not in the cervical glands. Since the quantity of material examined in California is only about 1 per cent. of the material examined

in Bombay, the figures from the former have not much influence on the figures from India. McCoy has once or twice seen rats dead from plague which did not show any of the characteristic pathologic changes. In these cases the presence of the plague bacillus was established by animal inoculation.

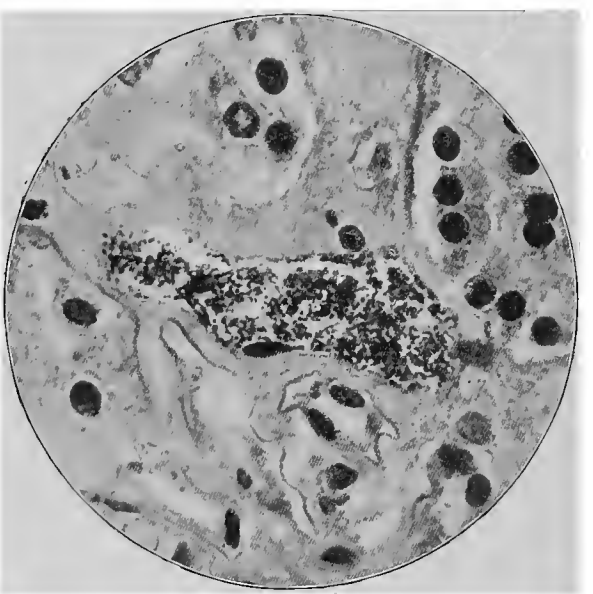
Plague-infected rats generally die in a few days, but sometimes, though rarely, the plague runs a chronic course. The Indian Plague Commission encountered a considerable number of these chronic cases in two villages in the Punjab Province.

Animals Susceptible.—According to Simpson, Hunter, and others, rats are by no means the only animals susceptible to natural plague infection; these authors also mention *pigs, calves, sheep, monkeys, geese, ducks, turkeys, hens, pigeons,* and *quails.* It is, however, very probable that this claim is not quite correct, and that other members of the group of hemorrhagic septicemia bacilli (Bacillus bipolaris) have been mistaken for the Bacillus pestis. Other rodents which, according to Blue, have been shown to harbor the Bacillus pestis, and to be a subject to plague infection, are the tarbegan, a species of arctomynæ, found in Siberia; the marmot, a hibernating rodent of India and China; the marmot of Thibet, the tree squirrel, and the California ground squirrel (Otosperphilus beecheyi). According to Blue and Wherry four plague-infected ground squirrels were found in California during the summer of 1907, and it was possible to show that several persons had contracted plague directly from this animal.

Spreading of the Infection.—Plague infection in man and animals is generally a wound infection, near which most cases present plague buboes. A certain percentage of cases are probably due to inhalation and lead to the *pneumonic type.* It is now claimed, particularly upon the basis of the early investigations of Simond and the more recent and extensive work of the Indian Plague Commission and the Advisory Plague Committee that the *rat flea* is responsible for the spread of plague from rat to rat and from rat to man. This flea most common on rats in tropical and subtropical countries has been described by Rothschild as *Pulex chœops.* About the same time the author, in ignorance of Rothschild's work, independently found and described the rat flea in Manila under the name of *Pulex philippinensis.* It is now claimed that the rat flea will frequently bite man, although in the author's experiments it did not. If plague is conveyed from rat to rat by the flea, it is certainly strange that most of the buboes in the rats in India, *i. e.*, 75 per cent., are found in the cervical region, which would indicate that fleas bite rats most commonly on the head and not on the body. The very frequent occurrence of the cervical bubo was formerly explained on the assumption that rats generally infected themselves by small abrasions or wounds in the mouth. It is also well known that even among well-fed white rats the living eat the dead of their own species.

Plague Bacilli in the Blood.—It appears that plague in rats generally leads to an early true septicemia, that is, to an invasion of the blood and a multiplication of the infecting bacilli. The Indian investigators in experimental rat inoculation have found as many as 1,000,000,000 bacilli per cubic centimeter of blood. This exceptional figure was, however, found only twice; other figures were as low as 10 to 100 per cubic centimeter. While one thousand million bacilli appears like an excessively high figure, it is not really so in view of the fact that one cubic centimeter of rat's blood contains 10,000,000,000 red blood corpuscles, and that in some of the other hemorrhagic septicemias, particularly those in birds, the bipolar bacilli are often many times in excess of the number of red blood corpuscles.

FIG. 126



Bacterial embolism in a vessel of the kidney (centre of field). Human infection by the bacillus of buhonic plague. (Author's preparation.)

Plague in Man.—According to the author's observations, this is generally not a septicemia but a local infection with a general toxemia. If blood in amounts of several cubic centimeters is obtained from plague patients and properly incubated in fluid culture media a growth is often obtained, but this may be due to the presence of a very few bacilli, and it only proves that a few live bacilli were in the blood at the time it was taken. In a true septicemia, however, a multiplication of the bacteria in the blood must be demonstrable. True hemorrhagic plague septicemia and pyemia with the formation of bacterial emboli

occurs in man occasionally, as it frequently occurs in rats, both naturally and experimentally.

Latent Plague.—It was Hunter, of Hongkong, who undertook to explain the seasonal appearance of plague among animals and man by attempting to show the existence of a latent form of plague in which small numbers of bacilli were present in the blood, but for the time being no multiplication nor development of symptoms of disease. From such latent cases outbreaks of epidemics might originate. Such conditions, of course, prevail in Texas fever and trypanosomiasis of cattle. Herzog and Hare, investigating this question in the Philippine Islands, have shown that latency in plague does not occur.

Morphology and Staining Properties.—The plague bacillus was discovered simultaneously and independently in 1894 by Kitasato and Yersin.

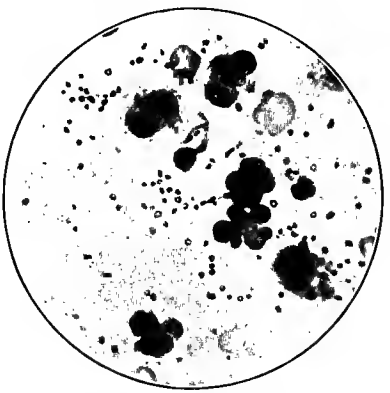
It is relatively variable in morphology, a fact which it is important to remember in connection with the bacteriologic diagnosis of the disease. In postmortem smears prepared from a recent non-suppurating primary bubo, a pneumonic focus, the spleen, and occasionally from other internal organs of both man and rats, numerous plague bacilli are generally found. The simplest staining method for plague bacilli in smears and in pus, necrotic material, etc., is dilute carbol-fuchsin (1 part of the original stain to 5 to 10 aqua destillata) for twenty to forty seconds and then washing freely in water.

In smears made from the organs, the plague organism appears as a rather short, plump bacillus, being 1.5 to 1.75 micra long and 0.5 to 0.75 micron thick; generally the proportion of width to length is as one to two. Individuals considerably longer than 1.75 micra are occasionally seen. The bacilli are generally single, occasionally diplobacilli, and very rarely in short chains. In smears which have been fixed in absolute alcohol and properly dyed the bacilli are not uniformly colored, but show a distinct polar staining. Frequently the entire periphery is stained and only the centre left uncolored. Other forms, differing in certain respects from the above description and not representing the most characteristic type, are so frequently found in smears that the student must thoroughly familiarize himself with them. These are elliptical, egg-shaped, or almost spherical forms, which show only a very narrow peripheral staining, or do not stain at all, giving the appearance of empty shells, which in all probability is the case, since they are most commonly found in older buboes. Various involution forms are also frequently seen in smears from cases which have succumbed but a few days after sickness. They appear like yeast cells, and are either quite hazy and indistinct or club-shaped and irregular in outline. In cover-glass preparations from pure cultures the bacilli are not so characteristic. Plague bacilli from pure cultures, particularly from the water of condensation of agar tubes, or from bouillon, frequently show shorter or longer chains, in which dividing lines between the individual bacilli are so indistinct as to

cause them to appear like filaments. Involution forms are also liable to present themselves early in a similar manner even on favorable media.

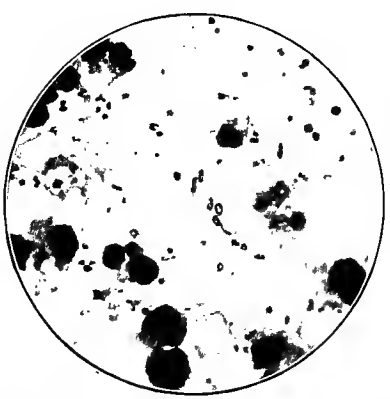
The plague bacillus is decolorized by Gram's method. When grown in the animal body the bacillus possesses a capsule, which,

FIG. 127



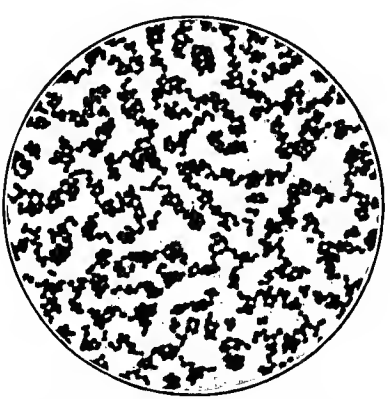
Postmortem smear from the spleen in a case of bubonic plague, showing polar staining. (Author's preparation.)

FIG. 128



Postmortem smear from a case of plague pneumonia, showing polar staining. (Author's preparation.)

FIG. 129

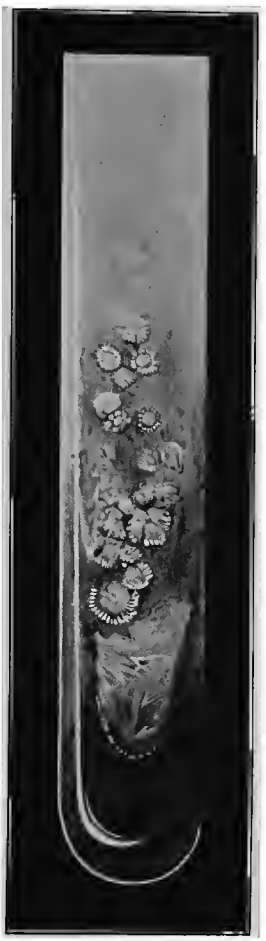


Cover-glass preparation from a twenty-four-hour-old agar plague culture, stained with dilute carbol-fuchsin, showing very small bacilli, with rounded ends. (Author's preparation.)

however, is difficult to demonstrate unless thin spreads are prepared and fixed with great care in alcohol. There is nothing characteristic in the capsule, so its exhibition will not assist in the microscopic diagnosis. The bacilli are not motile, do not possess flagella, nor

has spore formation been observed. Even though bodies somewhat resembling spores are occasionally seen in the bacilli they are not genuine spores, because such bacilli are not more resistant to heat, antiseptics, etc., than the other pest bacilli.

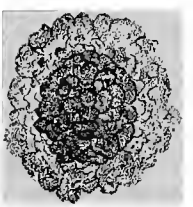
FIG. 130



Culture of plague bacillus on agar, five days' growth, fixed with formalin vapor. (Author's preparation.)

Cultural Properties.—The plague bacillus grows on all ordinary laboratory culture media, best on such as are faintly alkaline. Even a minor degree of acidity as well as a higher degree of alkalinity prevents development. It develops at temperatures ranging from 5° to 38° C., and in artificial media best at 25° to 30° C. It is almost strictly, though perhaps not absolutely, *obligate aërobic*. As a rule, it develops on artificial media only in the presence of free oxygen; some observers, however, have occasionally seen a weak growth in its absence. When a favorable solid culture medium (agar or gelatin, slightly alkaline) is inoculated from the organs (bubo, spleen, etc.) of a plague case the development of the bacilli is at first generally quite slow, and frequently very little can be seen with the naked eye in the first twenty-four hours. After this time a typical picture may appear in a considerable number of cases, and it is always present after forty-eight hours. The

FIG. 131



Colony of plague, gelatin plate culture, forty-eight hours old, showing dark elevated centre and transparent homogeneous marginal zone. (Author's preparation.)

surface of the agar or gelatin shows small, delicate, round, moist, dew-drop-like colonies. They are light gray in reflected light and grayish white in transmitted light. If these colonies are inspected with a hand lens or with a low power of the compound microscope they show an elevated, finely granular, rounded centre and a perfectly transparent, very thin, flat marginal zone. The colonies, on the whole,

are circular, but the transparent marginal zone early shows a somewhat irregular boundary line. If a young plague culture is touched with a platinum loop it is found to be viscous and sticky. It is, however, easily removed from the surface on which it grows.

The plague bacillus, as already stated, has a marked tendency to develop *involution forms* early in its growth. As first shown by Hankin, this tendency is most pronounced in cultures on a 3 to 4 per cent. salt agar, one of the most valuable media for the bacteriologic diagnosis of plague. It is prepared and standardized like an ordinary agar, except that it contains, instead of ½ per cent., 3 to 4 per cent. of common salt. Generally the greater number, or all, of the organisms from such a growth present themselves as large spherical bodies, looking very much like yeast cells; later, large, swollen club- or dumb-bell-shaped or irregular forms make their appearance. The most typical and most constant form grown on a 3.5 to 4 per cent. salt agar, after twenty-four to forty-eight hours, is the yeast-like, large, spherical plague organism. No other microörganism forms this type so promptly and regularly on salt agar that it might be confounded with the plague bacillus. Hence, it is advisable at the autopsy in a suspected case of plague to inoculate besides gelatin plates also ordinary agar tubes, bouillon flasks, and salt-agar tubes or plates. In *bouillon flasks* the bacilli, at temperatures between 30° to 35° C., show a finely flocculent whitish, slowly increasing sediment after twenty-four hours. During the next twenty-four hours the flocculi extend upward from the bottom along the walls. A fine whitish ring of growth then forms on the surface, and in course of time covers it. If the flask is kept motionless and undisturbed, bands and strands of bacilli finally grow downward from the surface membrane. The contents of the flask now present an appearance somewhat resembling stalactite and stalagmite formations.

The plague bacillus does not liquefy gelatin or blood serum; does not ferment dextrose, levulose, lactose, or mannite; and grows sparingly on potato and in milk. It does not coagulate the latter.

Resistance.—The resistance of the plague bacillus is not great; it is about the same as that shown by other members of the group. At 70° C. the bacilli are safely killed within one hour; sunlight and drying destroy them within twenty-four hours; 1 per cent. lysol solution in ten to fifteen minutes; 1 per cent. caustic lime in ten minutes; 1 per cent. corrosive sublimate solution in half a minute; 1 per cent. hydrochloric acid solution in one minute; ½ per cent. sulphuric acid in five to ten minutes.

Vaccine and Serumtherapy.—Killed cultures of the plague bacillus have been prepared and used as vaccines by Yersin, Calmette, Borrell, Haffkine, and others. Haffkine's has been used extensively in India. Attenuated but live cultures have been used by Kolle and Otto and Strong. An antiplague serum has been prepared by a number of investigators by the inoculation of horses. Vaccination against plague

with dead cultures has evidently a valuable protective influence; the antiserum has not been very successful as a curative agency in cases of plague after they are sufficiently well developed for a diagnosis.

QUESTIONS.

1. What is a hemorrhagic septicemia? What are its most characteristic pathologic changes or lesions?
2. What kind of bacteria do occasionally; what kind generally lead to hemorrhagic septicemia?
3. Give the common features of the bacteria of the group of Bacillus plurisepticus (bipolaris).
4. Does the anthrax bacillus belong to this group? If not, why not?
5. What animal diseases are due to bacilli of the hemorrhagic septicemia group? What have they been called by the French writers? What is meant by the term Pasteurella?
6. Why are these organisms known as the Bacillus bipolaris septicus?
7. What was Pasteur's work in connection with the disease known as chicken cholera?
8. Describe the pathologic lesions of this disease.
9. In what part of the intestines are the changes most pronounced?
10. Describe the morphology and cultural properties of the Bacillus (bipolaris) avisepticus.
11. How can its virulency be increased; how can the organism be attenuated?
12. What animals are susceptible to the organisms?
13. What steps are necessary in establishing beyond doubt the diagnosis of fowl cholera?
14. Discuss the resistance of the Bacillus avisepticus and the effective methods of disinfecting infected hen houses.
15. How can active and passive immunity against fowl cholera be procured?
16. Does the fowl cholera bacillus form a soluble toxin?
17. Where does hemorrhagic septicemia of cattle occur?
18. Describe its pathologic lesions. What are the characteristics of the spleen in hemorrhagic septicemia and in anthrax?
19. Describe the morphologic and cultural properties of the Bacillus (bipolaris) bovisepticus.
20. Is the cornstalk disease of cattle due to this bacillus?
21. What is the cause of septic pleuropneumonia of calves?
22. What is the cause of hemorrhagic septicemia in sheep?
23. Why has there been a considerable confusion in the past as to the etiology and pathology of hemorrhagic septicemia of swine?
24. Give the other names for this disease.
25. What organism is the cause of swine plague?
26. Describe the most prominent pathologic lesions in a very acute case of swine plague.
27. What is the meaning of the terms petechiæ and ecchymoses?
28. What are the most prominent pathologic lesions in chronic swine plague?
29. Describe the Bacillus (bipolaris) suisepticus. Where is it found in acute, where in chronic cases?
30. What is the vacuole bacillus of Bang? What the Bacillus parvus ovatus of Loeffler?
31. Describe the cultural properties of the swine plague bacillus. What animals are susceptible to it?
32. Discuss the resistance of the organism.
33. What is a polyvalent immune or antitoxic serum?
34. How is the polyvalent antiswine plague serum of Ostertag-Wassermann prepared?
35. Under what other names is the equine disease pink-eye known?
36. What organism according to Lignière causes this disease?
37. Describe the most prominent lesions of an acute case of equine influenza.
38. What are the principal lesions in the pectoral form of the disease?
39. Describe the morphology and cultural properties of the Bacillus equisepticus of Lignière.

40. What animals are susceptible to the pathogenic action of the bacillus; how does natural infection occur?
41. Discuss the canine disease known as dog typhus or gastro-enteritis hemorrhagica.
42. What organism causes bubonic plague in man, rats, and other animals?
43. Define and describe a plague bubo.
44. Define the following terms: primary bubo of the second order; secondary bubo of the first order.
45. What lymph glands in rats are most commonly the seat of the primary bubo?
46. Name the lymph glands affected in the order of their frequency.
47. Describe the liver changes frequently found in rats dead from plague infection.
48. Describe the appearance of the subcutaneous connective tissue after removal of the skin.
49. What pathologic change in the pleura is very typical for rat plague?
50. What other animals in general are said to be susceptible to natural plague infection? What other rodents in addition to the rat are susceptible?
51. How is plague infection spread from rat to rat and from rat to man?
52. Is the general transmission by rat fleas proved beyond doubt? If not, why not?
53. Discuss the finding of plague bacilli in plague-sick rats.
54. Is plague in man generally a true septicemia?
55. What is latent plague? Among what animals or human beings does it occur?
56. Describe the morphology and staining properties of the Bacillus pestis.
57. Describe its cultural properties.
58. Discuss its resistance.
59. Have vaccine and serumtherapy been tried in plague, and how?

CHAPTER XX.

ANTHRAX BACILLUS.

Occurrence and Pathogenesis.—Anthrax, splenic fever, malignant pustule, carbuncle, woolsorter's disease, Milzbrand (German), charbon (French), is a disease of man and the lower animals which has apparently been known to mankind for thousands of years. It is probably the affection referred to in the Bible (Exodus, ix, 3–9), and it is mentioned by several of the classic Greek writers, including Homer. Its names are derived from the most obvious anatomic lesions, enlargement, softening, and congestion of the spleen (*splenic fever, Milzbrand*), and the widespread occurrence of acute passive congestion and acute hemorrhagic infiltration of the subcutaneous, subserous, and submucous tissues, which generally give to them a very dark brown or dark purple or sometimes an almost black color. The term *anthrax* is derived from the Greek word for coal, which is also the meaning of the French *charbon*. The designation *woolsorter's disease* is derived from the fact that the handlers of hides from cows or wool from sheep dead from anthrax often contracted the disease, which in this case usually assumes the local character of a malignant *pustule* or *carbuncle* or an *inhalation pulmonary affection*. The disease is caused by a pathogenic microörganism known as the anthrax bacillus. It is most common among cattle and sheep, but also occurs in man, horses, hogs, goats, deer, hares, buffaloes, dogs, cats, and very rarely among chickens, ducks, and geese. An anthrax epidemic among the wild animals of the zoölogical garden of Copenhagen has been described by Jensen, and a laboratory epidemic among guinea-pigs that contracted the disease from anthrax-infected peat has also been reported. The susceptible laboratory animals used in experimental work are mice, guinea-pigs, and rabbits. Gray rats are very slightly susceptible, white rats more so. Anthrax is prevalent particularly among cattle and sheep practically throughout the entire world. It has been found in Europe, Asia, Africa, Australia, and North and South America, but it is by no means equally distributed in all places, being much more prevalent in moist, marshy lowlands or prairies than in dry, rocky soil. It is endemic in favorable localities, where it finds its numerous victims year after year; it also occurs in sporadic outbreaks.

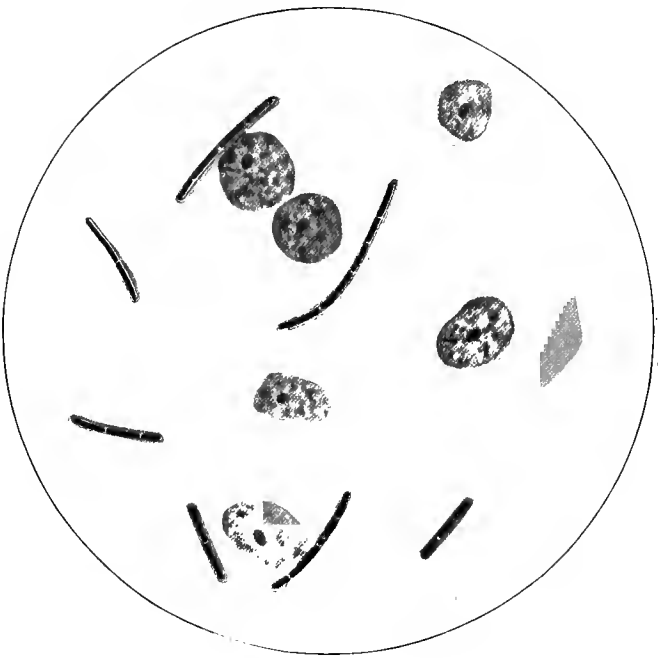
Pathologic Lesions.—The anatomical changes of anthrax are quite characteristic. The blood, on account of a lack of oxygenation due to toxic influences, becomes very dark and does not promptly and

sufficiently coagulate after death. Postmortem rigidity is not strong nor does it continue very long, and putrefactive changes with gas formation rapidly set in. Blood oozes from the mouth, the nose, and the anus. The mucous and serous membranes are enormously congested and show *petechiæ* and *ecchymoses*. Hemorrhages in the connective tissue are also found in various places. The subcutaneous and intramuscular connective tissues show an œdematous, gelatinous infiltration with hemorrhagic patches here and there. The lymph glands are swollen and edematous. The *spleen* is generally much swollen, much congested, its pulp very soft, and the capsule tense. It ruptures easily, and an almost fluid pulp mass may be discharged spontaneously. The liver and kidneys are swollen, congested, and, when cut into, discharge much dark blood and present a dull surface indicating parenchymatous degeneration. The lungs and the brain are likewise hyperemic and edematous. The mucosa of the intestines is swollen, dark, sometimes necrotic, often raised in patches by edematous and hemorrhagic infiltration in the form of ridges or globular masses. The blood, when examined microscopically, shows a very marked increase in the number of leukocytes (leukocytosis or hyperleukocytosis) and enormous numbers of anthrax bacilli. These, however, are found in the capillaries of the internal organs rather than in the larger vessels. Sometimes in very rapid so-called fulminant cases the anatomical changes are not as well marked as described above, because the fatal intoxication has been so rapid that the characteristic anatomical changes have not had sufficient time to develop. The spleen, however, is generally very congested, enlarged and softened in all cases.

The Discovery of the Bacillus.—Anthrax bacilli were first seen in the blood of animals dead from the disease by Pollender and Brauell. The latter also made some successful inoculation experiments on animals. These were later repeated and extended by Davaine, who had previously written concerning the rod-shaped bodies in the blood of animals which had died from splenic fever. The etiology of anthrax was first firmly established by pure culture and animal experiments by Robert Koch, in 1876, who was also the first to discover the spores of the bacillus.

Morphology.—The Bacillus anthracis is a rod-shaped bacterium 1.5 to 10 micra long (average length, 4 micra), and 1 to 1.25 micra thick. It is, therefore, one of the largest pathogenic bacteria. It is not motile. The exact morphology of the bacilli can best be studied in the blood of animals which have just died or are in the agonal stage of the disease. The bacilli appear in large numbers in the blood ten to twelve hours before death. Examination of the unstained blood of a mouse or guinea-pig in a moist cover-glass preparation shows transparent, colorless, cylindrical rods which have no motility whatever between the erythrocytes. Individual bacilli or short chains of two, three, or four bacilli occur. Long chains or spores are never seen in

PLATE VI



Bacillus of Anthrax in the Blood of a Cow.

Natural infection. Capsules stained with eosin.

fresh blood preparations. If the blood or the juice from the spleen is obtained a considerable time after death, when putrefactive changes and the admission of air has set in, longer chains and spores may be found.

Staining Properties.—The anthrax bacillus stains well with the ordinary watery anilin stains, and is Gram positive. When fixed and stained in the usual manner the bacilli show certain features not seen in the fresh, unstained specimen. The ends of the bacilli appear sharper and are not so regularly cylindrical as before, but slightly swollen, and show a little excavation of the outer surface, so that a small lenticular empty space is formed between two individual bacilli of a chain. A chain of anthrax bacilli after it is stained looks very much like a stick of bamboo. In the stained blood or splenic juice preparation the anthrax bacillus shows a gelatinous capsule, which, however, is not present in bacilli obtained from pure cultures on the ordinary artificial media. Johne has devised a special method for clearly demonstrating the anthrax bacillus capsule in blood preparations. His method is as follows:

1. Prepare a blood smear on a cover-glass, allow it to become air dry, and fix by drawing carefully and rapidly through a flame. Do not overheat or the capsules will be burned.
2. Apply to the fixed cover-glass 2 per cent. watery gentian-violet solution and heat slightly for one-quarter to one-half minute over a flame.
3. Wash rapidly in water.
4. Apply for six to ten seconds a 1 to 2 per cent. solution of acetic acid.
5. Wash in water and mount in water (not in Canada balsam) on a slide and examine while moist.

The capsules may also be stained by any of the other methods for exhibiting this plasmatic envelope.

Spores.—Spores can easily be demonstrated in cultures raised on artificial culture media in the presence of oxygen. As far as is known, the organism never forms spores in the absence of free oxygen, and they are never seen in the living blood. They are formed in artificial media at blood temperature after eighteen hours, at 18° C., after fifty hours; at 14° C. spores are no longer formed. The spore of the anthrax bacillus is found in the middle of the rod, but since the protoplasm of the latter soon degenerates and perishes after sporulation, it may appear as if this were not the case. Every bacillus forms a single spore, which is not perfectly spherical but somewhat egg-shaped or elliptical. These spores possess a very tough membrane; some observers even claim that the anthrax spore has two membranes. The following method of staining the spores is recommended as the most trustworthy:

1. Take several platinum loopfuls of material from an anthrax growth on a solid medium and rub it up well with a 0.85 per cent. salt solution.

2. Mix 1 or 2 c.c. of this emulsion with an equal amount of Ziehl's carbol fuchsin and place in a watch-glass or small beaker.

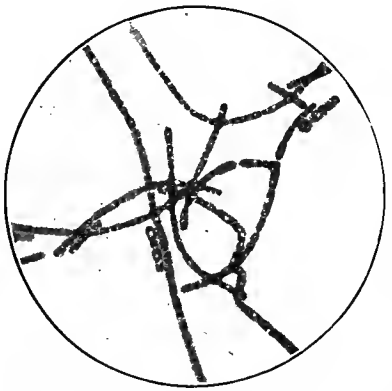
3. Heat over a small flame for about six minutes, until vapor rises.

4. Spread some of the mixture upon a cover-glass or slide, allow the preparation to become air dry, and fix by drawing twice through a flame.

5. Decolorize for one or two seconds in 1 per cent. sulphuric acid watery solution; wash in water, counterstain in watery methylene blue for three or four minutes.

As a result of the stain the spores show red and the bacilli blue. The best culture medium for the production of an abundant spore formation is boiled sterilized potato, cut into halves or disks, and kept at temperatures between 31° and 37° C. Another excellent method for obtaining abundant sporulation consists in pouring bouillon containing anthrax bacilli on agar plates. When spore formation is studied directly under the microscope the following changes are seen. There first occurs a clouding of the previously clear protoplasm of the bacillus, the latter forming fine granules and appearing as if it had been dusted. Then larger, highly refractive granules appear, and these, probably by uniting with each other and with other substances of the bacillus, form the round or slightly oval spore.

FIG. 132

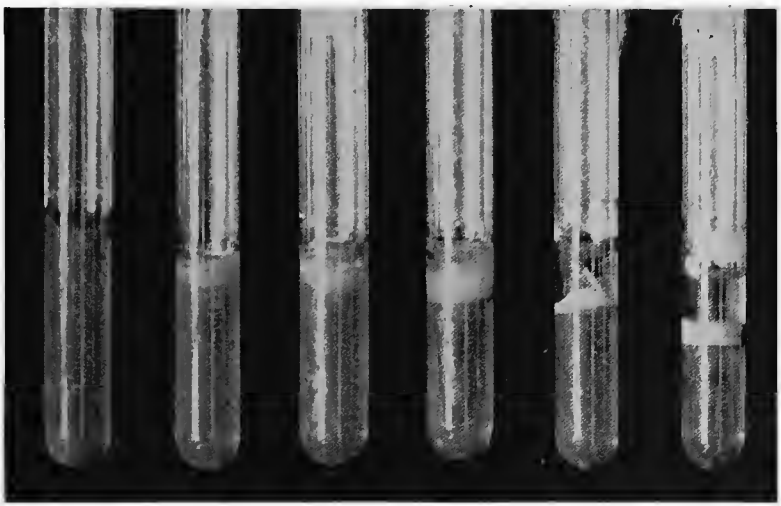


Anthrax bacillus, spore formation × 1000. (Author's preparation.)

Germination of Spores.—Spores or sporulating bacilli under favorable conditions, either in nature, in the body of a susceptible animal, or in a suitable culture medium, develop again into young bacilli. The tough spore membrane swells up and becomes elongated, the spore itself within the membrane loses its shining refractive appearance, and a hole or rupture appears in the membrane through which the elongated spore, having now assumed the shape of a young bacillus, makes its exit. Occasionally a short bacillus with a round, empty capsule at one end is seen in preparations from anthrax cultures, giving the appearance of a bacillus with a spore at one pole, such as is found in the case of the sporulating tetanus bacillus. With the anthrax bacillus, however, this appearance is deceptive and in reality only indicates a young bacillus and an empty spore membrane or capsule at one end. Germination of spores, after they have been brought into a favorable culture medium, and if kept at temperatures between 30° and 37° C., begins generally after eight hours.

Asporogenous Anthrax Bacillus.—A variety of the anthrax bacillus which no longer forms spores may be raised by adding certain antiseptics, such as carbolic acid in the proper proportion to the culture medium. After these artificially produced varieties have once lost the property of forming spores, it is not regained even when they are transplanted to culture media free from antiseptics.

FIG. 133



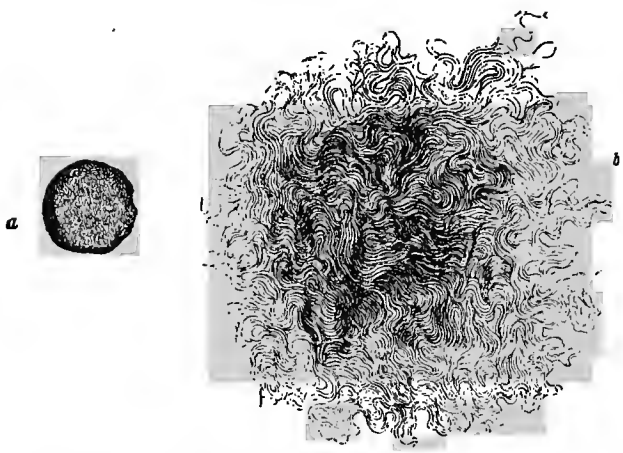
Six cultures of antbrax bacillus, showing increase in liquefaction, with age. (Author's preparation.)

Cultural Properties.—Artificial cultivation of the anthrax bacillus is very easy. It can be accomplished from infected blood, discharges or tissues by the plate method or by preliminary inoculation of the infected material into mice or guinea-pigs. The organism grows well on all the ordinary laboratory culture media. It has a wide range of temperature (between 15° and 43° C.), in which it can grow and multiply, the most favorable temperature being between 30° and 40° C. It not only grows well on the ordinary laboratory media, but also on sterilized disks of cucumbers, in the sterilized and neutralized juice of pears, onions, and beets; in urine, provided it is not too alkaline, and particularly well in urine containing albumin. It is readily seen that an organism thriving at such a great range of temperature and in so great a variety of media easily finds opportunity to multiply in the outside world as a saprophyte.

Gelatin.—On gelatin plates the organism first forms small, round, grayish-white colonies upon the surface; these rapidly begin to liquefy the culture soil. In the depth of the medium the young colonies are seen as elliptical, slightly brownish, granular, small points or disks,

which do not grow as well as the surface colonies. The latter soon spread out into larger spots, with a still generally spherical but more irregular outline, from which bundles of filaments and cords formed by the bacilli project. The whole formation then somewhat resembles a tuft of wool or the serpent-covered head of the mythological Greek Medusa, as can be demonstrated in stained specimens, prepared by the so-called impression method (Klatsch-Präparat, German). Such preparations are made by pressing a perfectly clean dry cover-glass upon a surface colony on a gelatin plate and then carefully lifting it off with forceps. The impression on the cover-glass is then air dried, fixed, and stained in the usual manner.

FIG. 134



Colonies of bacillus anthracis upon gelatin plates: *a*, at the end of twenty-four hours; *b*, at the end of forty-eight hours. × 80. (F. Flügge.)

A *gelatin stick culture* of anthrax is very characteristic. The growth first develops on the surface and along the stab, because the bacillus requires oxygen in artificial cultures. The very surface growth, for this reason, is most luxuriant, and the pellicle formed is irregular and uneven. Along the stab itself a radiating or arborescent effect is produced, making the culture at first somewhat resemble an inverted pine tree. Liquefaction of the gelatin is most pronounced at the surface where the growth is most abundant. Some of the liquefied material runs down into the canal of the stab and some of the fluid is lost by evaporation, causing a cup-like or cone- or funnel-shaped depression to form on the surface. After several days when the growth has become more abundant the entire upper stratum of the gelatin may be liquefied and the growth itself sinks to the bottom of the fluid layer.

Agar and Other Media.—On agar plates the development is similar to that on gelatin plates. There is, of course, no liquefaction, because

the peptonizing ferment of bacteria digests and fluidifies gelatin, blood serum, egg-albumen, etc., but not agar. *Agar stick cultures* also resemble gelatin stick cultures, except for the absence of liquefaction, and the inverted pine-tree effect is often well produced. On *agar slants* gray, granular, shiny growths develop, which later become uneven and folded and cannot be very easily removed from the surface for microscopic examination without the use of some force with the platinum loop. On *potatoes* the anthrax bacillus grows very luxuriantly, forming a whitish, dull, shining mass, and the spore formation is generally very abundant and seen early. The growth on *blood serum*, as on gelatin, leads to liquefaction. *Sterile milk* is first coagulated by the anthrax bacillus, later the precipitated casein is liquefied. Fresh unpasteurized or unsterilized milk is not a favorable culture soil, because the developing lactic acid bacteria prevent the growth of anthrax bacilli causing them very soon to die out completely, unless spores have previously been formed. In *bouillon* the growth of the heavy non-motile bacilli leads to the formation of slimy flocculi which sink to the bottom. From this sediment filamentous masses arise, but the upper layers of the fluid generally remain clear.

Resistance of the Anthrax Bacillus and its Spores.—The vegetative form of the anthrax bacillus is not very resistant to inimical germicidal and physical influences, but the spores are much more resistant than those of most other pathogenic bacteria; tetanus spores, however, withstand moist heat better than anthrax spores. Anthrax bacilli are killed when heated to 55° C. for forty minutes if contained in fresh blood, and in one hour if heated to 50° C. A spore-free bouillon culture is killed if exposed for five and one-half minutes to 65° C., and in three minutes if heated to 75° C. Dry air of 140° C. kills anthrax spores only after three hours' exposure; steam of 95° C. after ten minutes; steam of 100° C. after five minutes. Low temperatures as they ordinarily occur in nature in countries of medium latitude have no appreciable effect upon either bacilli or spores. Direct sunlight kills bacilli and spores in a comparatively short time, particularly when the air has free access while the rays are acting upon the organism. Bacilli are easily killed by a 1 to 1000 solution of corrosive sublimate, and even spores are killed after a few hours, but this powerful action is only obtained when the solutions act upon dry bacilli or spores or when the latter are suspended in a watery solution. In the presence of albumin, as in blood, the action of mercury bichlorid is untrustworthy. Carbolic acid in watery solutions acts weakly toward spores. Salting and curing of meat kill anthrax bacilli in from two to four weeks, but has no effect upon the spores. The most markedly antagonistic bacterium to the anthrax bacillus is the Bacillus pyocyaneus. If a mixed culture of anthrax and pyocyaneus is made the latter will develop and the former will die out. This effect is probably due to a ferment secreted by the Bacillus pyocyaneus, and known as pyocyanase. The streptococcus

and staphylococcus are also antagonistic to anthrax, but to a less degree.

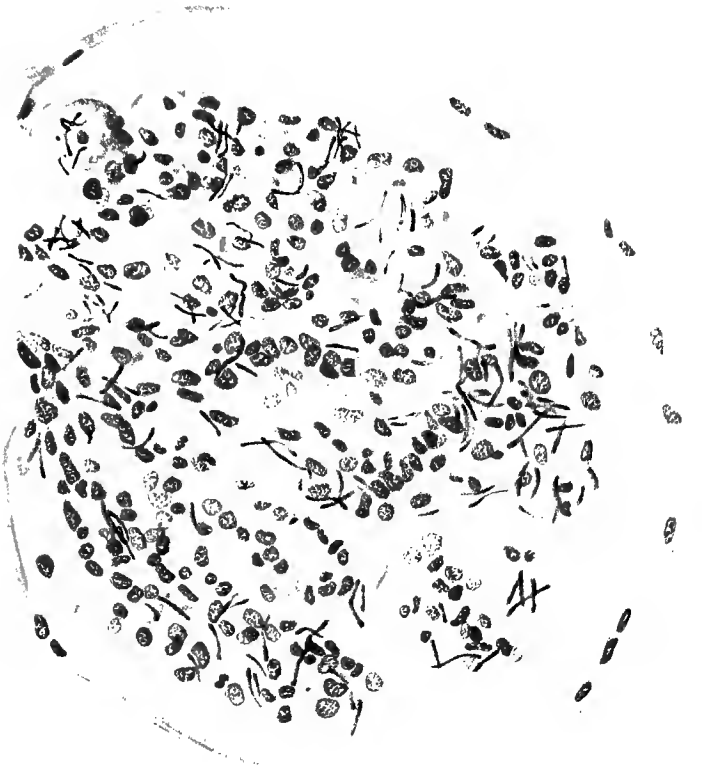
Modes of Infection under Natural Conditions.—Robert Koch has done more than anyone else to show how anthrax is spread by the secretions of sick animals and from cadavers. The excreta and the hemorrhagic discharges from the mouth, nose, and anus contain anthrax bacilli, which are likely to invade barns and pastures. Bacilli are also spread when cadavers are skinned and opened and when they are buried superficially. Spore formation occurs under all these conditions. While anthrax bacilli themselves easily perish in the presence of numerous putrefactive bacteria, spores when once formed are very resistant and can survive in moist and dry soil for two or three years, in water for seventeen months, and in cesspools for fifteen months. After spores have formed they may be spread by infected hides and by hay collected from infected pastures and stored in barns. Ravenel was able to show that anthrax was spread by a tannery working with infected hides; others have demonstrated the persistence of anthrax spores for twelve to twenty years in sand in which an anthrax cadaver had been superficially buried. Kitasato, on the other hand, demonstrated that there is no spore formation in an infected cadaver buried as deep as six feet. By far the most common route of infection is by the ingestion of food contaminated with anthrax spores. The vegetative form, *i. e.*, the bacillus, when taken into the healthy stomach, is probably always, at least generally, destroyed by the gastric juice; but the spores pass the stomach and germinate in the intestines, where the bacilli multiply and break through the mucous membrane into the general circulation. Domestic animals also show an inoculation anthrax through wounds of the buccal mucosa or skin. Pulmonary inhalation anthrax probably does not occur among domestic animals. Intestinal anthrax is not always contracted in the pasture, and may, during winter, be contracted from stored infected hay.

It has been observed that anthrax sometimes occurs sporadically in a single animal or a few individuals of a herd while the great majority escape entirely. Sobernheim believes this to be due to a mild unnoticed form of anthrax common among cattle and sheep in anthrax-infected neighborhoods, which leads to immunization of the majority of the herd by a natural mild infection.

Anthrax in man occurs chiefly in persons who are exposed to the disease by means of infected hides, wool, and other similar carriers. The most common form is the local wound infection, the *anthrax carbuncle;* the *inhalation pulmonary form* follows, and the *ingestion intestinal form* is least common. The cutaneous form usually ends in recovery, while the pulmonary and intestinal forms, as a rule, lead rapidly to a fatal termination.

Diagnosis.—While the clinical symptoms and anatomical findings in anthrax are quite characteristic, they more or less resemble those of emphysematous anthrax or black-leg, malignant edema, hemor-

PLATE VII



Kidney of Steer. Anthrax Bacillus Infection.

Zeiss objective 4 mm.; compens. ocular No. 6.

rhagic septicemia, etc., and for this reason, microscopic and laboratory examinations are necessary for a trustworthy diagnosis. This is particularly necessary in the case of a fresh outbreak or when the disease appears in a district which has previously been free from anthrax. Sufficient evidence for a trustworthy diagnosis can often be obtained from a microscopic examination of the blood, particularly in the last stages of the disease while the animal is still alive or within a few hours after death before the body has been opened. In the former case blood may be obtained from the ear by a slight cutaneous incision. When a postmortem examination is made it is best to take the blood from the spleen. Blood obtained in this way from the small peripheral vessels or the spleen may show the non-motile, sporeless, cylindrical, square-ended, capsule-possessing bacilli in short chains. When putrefaction sets in, putrefactive bacteria which closely resemble the anthrax bacillus appear in the cadavers of cattle and sheep. Under unfavorable conditions, cultures must therefore be prepared or animal experiments made. When there is access to neither a microscope nor laboratory facilities in cases of suspected anthrax in cattle and sheep, the material should be prepared for subsequent examination in a laboratory in the following manner:

1. Prepare a few microscopic slides by spreading some blood from the animal on them in thin layers.

2. Clean a wide-mouthed bottle by washing it with an antiseptic solution and then with alcohol. Invert the bottle and allow it to dry. Place in it a portion of spleen, or if the cadaver has not been opened, a portion of the ear, and close the bottle tightly with a cork, the inner end of which has been sterilized by burning.

3. Take a potato, clear it of earth, wash and scrub it externally with a 1 to 1000 solution of corrosive sublimate. Boil it well in water in any suitable covered vessel. After the water has cooled, take out the potato, dry it externally with a clean cloth. Cut it into halves with a knife previously heated over a flame. In this manner a perfectly sterile potato is obtained. Allow some blood to flow on one of the flat surfaces, place the halves again face to face, and wrap up the potato, if possible, in clean aseptic gauze. Place the potato in a suitable container (glass vessel, clean tin cup, empty gauze box, etc.).

4. Send the blood slides, piece of spleen, and inoculated potato to the laboratory by the quickest possible route without loss of time.

The trained bacteriologist is immediately able to stain and microscopically examine the blood slides. He can soon examine the culture on the potato and from it and the piece of spleen inoculate mice and guinea-pigs. These animals are very susceptible, and will show, in one or two days, a typical anthrax infection if the material actually contained anthrax bacilli. The inoculation of the suspected material is made subcutaneously from an emulsion, or a piece is introduced into a small subcutaneous pocket, made with a slender scalpel or a pair of small scissors. Mice and guinea-pigs infected in this manner

soon become quiet, refuse to take food, and after twenty-four to forty-eight hours suddenly fall over with some convulsions and die. Death is due to paralysis of the respiratory centres and asphyxia. The post-mortem examination shows an edematous gelatinous infiltration at the place of inoculation; a congested, soft, and very much enlarged spleen; and dark blood which, under the microscope, exhibits the bacilli as described above. These findings make the diagnosis of anthrax absolute.

Prophylaxis.—Prophylaxis against the spreading of anthrax should in the first place concern cadavers. The best method of disposal is to bury them in quicklime at or near the place where the animals have died without loss of time and without opening or skinning them. The bodies should be interred at a depth of five to six feet, so that they may be completely covered. Sick animals in which there is little prospect of recovery should be killed, but not by bleeding, and the cadaver should be treated as recommended above. The place of burial should be fenced in for several years to prevent cattle and sheep from grazing there. Burning of the cadavers is also strongly recommended, but facilities for this are rarely to be found at the place of death, and transportation to a distant place, particularly if the animal is skinned, involves a probable spreading of the infection. In Germany the law prohibits the skinning of animals sick with or dead from anthrax. If, for economic reasons, the hides are to be saved, they and the knives used in the skinning must be properly disinfected; the latter by boiling in water, the former by immersion for a number of hours (not less than twelve) in strong carbolic acid, creosote, lysol, or other similar solution. The process of tanning does not kill anthrax spores.

Barns, stalls and utensils which may have been contaminated from anthrax-infected animals must likewise be disinfected.

Vaccines and Serumtherapy.—Vaccination for the protection of animals against anthrax has been employed for a considerable time. The vaccine used contains live but attenuated and sporeless anthrax bacilli. There are various methods of decreasing the virulency of anthrax bacilli, such as cultivation in the presence of small amounts of antiseptics (carbolic acid, bichromate of potash, sulphuric acid), cultivation in the blood serum of immune or immunized animals, cultivation under higher atmospheric pressure, and cultivation in successive generations at comparatively high temperatures. The latter method is generally employed in practice to obtain vaccines for the protective inoculation of domestic animals. The higher the temperature under which the anthrax bacillus has been grown and the longer the cultivation has been continued the more attenuated the organism becomes; but in order to get a truly protective vaccine the attenuation must not be carried too far. The method most commonly employed is that of Pasteur, and the vaccines used are prepared as follows:

Vaccine No. 1 (the more attenuated, weaker vaccine) is a bouillon culture of anthrax bacilli grown in successive generations in the incubator at a temperature of 42° C. for twenty-four days.

Vaccine No. 2 (the stronger, less attenuated vaccine) is a bouillon culture of the anthrax bacillus grown in the incubator at the same temperature (42° C.), but only for twelve to fourteen days.

Vaccine No. 1, in small, proper doses, should kill mice, but not guinea-pigs or rabbits. Vaccine No. 2 in the same doses should kill mice and guinea-pigs, but not rabbits. Non-attenuated cultures in the same doses kill mice, guinea-pigs, and rabbits.

To protect domestic animals against a subsequent anthrax infection they are first inoculated with the weaker vaccine, No. 1, and after an interval of twelve to fourteen days with the stronger vaccine, No. 2. The dose of both No. 1 and No. 2 is $\frac{1}{4}$ c.c. for horses, mules, and cattle, and $\frac{1}{8}$ c.c. for sheep and goats. The vaccines are injected subcutaneously, behind the shoulder in cattle, inside the thigh in sheep and goats, and on the side of the neck in horses. It is best to inject No. 1 on one side and No. 2 on the other side.

Vaccination of a large number of animals is attended with some loss. Even with every precaution, and with carefully prepared vaccines used properly, the losses are about one animal in several hundred. With improperly prepared, insufficiently attenuated cultures heavy losses have occurred. Every fatal case may become the source of spreading a virulent infection. The dispenser and user of the anthrax vaccine must remember that cadavers of animals dead of anthrax vaccination must be disposed of in the same manner as the cadavers of animals dead of the natural infection and that barns and environments must be carefully disinfected.

According to the statistics compiled by Hutyra, the following vaccinations according to Pasteur's method were made in 1889-1890 in Hungary, with the following results:

Head of horses vaccinated	39,506
Percentage dead of anthrax between first and second vaccination	0.1
Percentage dead of anthrax during first year after second vaccination	0.09
Head of cattle vaccinated	718,266
Percentage dead of anthrax between first and second vaccination	0.02
Percentage dead of anthrax during first year after second vaccination	0.02
Head of sheep vaccinated	1,247,231
Percentage dead of anthrax between first and second vaccination	0.26
Percentage dead of anthrax during first year after second vaccination	0.33

Anti-anthrax Serum.—That the serum of animals actively immunized against anthrax possesses specific immune bodies has been shown by Sclavo, Marchoux, and Sobernheim. These investigators have prepared an antiserum which they have used for prophylactic and therapeutic purposes. The antiserum is prepared by hyperimmunizing

such animals as sheep, horses, and donkeys. These animals first receive vaccine No. 1 and No. 2 in the usual doses and intervals. About two weeks after the last vaccination they receive an injection of a more virulent culture. This injection is repeated in from ten to fourteen days, always with cultures of greater virulence, or with increasing doses of a virulent culture. All injections are made subcutaneously, not intravenously. After two or three months of this treatment the serum of the hyperimmunized animals has obtained a high value. If injected in doses of 20 to 25 c.c. into horses, cattle, and sheep it produces a passive immunity which will protect the treated animal against anthrax infection for several weeks, or perhaps at best for two or three months. The serum has also proved effective as a curative agent after anthrax infection has taken place, but it must then be used in larger doses of 30 to 150 c.c. In cases of human local infections (carbuncle) the antiserum has been used in doses of 30 to 40 c.c. injected subcutaneously in three or four different places on the body; in very bad cases another set of injections of the same doses should be made after twenty-four hours. It has also been used in bad cases in 10 c.c. doses as an intravenous injection. Sobernheim has worked out and used on animals, with excellent results, a combined, simultaneous method of active and passive immunization. He uses vaccine No. 2 ($\frac{1}{2}$ c.c. for horses and cattle, $\frac{1}{4}$ c.c. for sheep and goats), and injects it subcutaneously into one side of the animal, while the other side receives 4 to 5 c.c. of the antiserum. The advantages of this combined method are that it requires only one treatment and that the animals are fully protected after ten to twelve days. It appears that the *simultaneous method* is absolutely safe and that it has never led to any fatalities following its proper use. The last 50,000 vaccinations, according to Sobernheim, were made without a single death.

It is well to state, however, that nothing is known at present concerning the mechanism of the action of the antiserum. It does not contain an antitoxin, because the anthrax bacillus does not form soluble toxins. It does not increase phagocytosis and it does not act as a bacteriolytic agency. It acts in some way as a bactericidal antiserum. It seems to prevent the anthrax bacilli from leaving the place of injection and entering into and multiplying in the general circulation. It causes them to die out in the subcutaneous tissue after a comparatively short time, though by no means immediately.

QUESTIONS.

1. What other names have been given to the disease anthrax? How long has it been known to mankind?
2. What animals are susceptible to it?
3. Describe the pathologic changes of anthrax.
4. What is meant by the parenchymatous degeneration of an organ?
5. What is a leukocytosis or hyperleukocytosis?
6. What is meant by a fulminant case of anthrax or of an infectious disease in general?
7. Describe the anthrax bacillus (*a*) in the blood of an animal dead from the disease; (*b*) from a pure culture.
8. Describe some methods of staining (*a*) the capsule; (*b*) the spores of the Bacillus anthracis.
9. Describe the changes in the bacillus in sporulation.
10. Describe the changes in the spore in germination.
11. What is an asporogenous anthrax bacillus?
12. Describe a gelatin stick culture of the anthrax bacillus.
13. What is an impression or Klatsch preparation of an anthrax colony? How does it look?
14. Describe the growth of the anthrax bacillus in bouillon, on potatoes, in milk.
15. Discuss the resistance of the anthrax bacillus and its spores.
16. What bacterium is very antagonistic to the anthrax bacillus?
17. How do cattle and sheep generally contract anthrax?
18. How do men contract the disease?
19. Describe the steps to establish beyond doubt the diagnosis of anthrax.
20. How is anthrax-suspected material prepared for forwarding to a distant laboratory for diagnosis?
21. How should anthrax cadavers be disposed of?
22. Describe the method of Pasteur used in the preparation of the anthrax vaccines.
23. Describe and discuss their use in the protection of cattle and sheep.
24. How is the anti-anthrax serum prepared?
25. What is the simultaneous method to protect animals against anthrax? What are its advantages?
26. Describe the different toxins of anthrax and their action.

CHAPTER XXI.

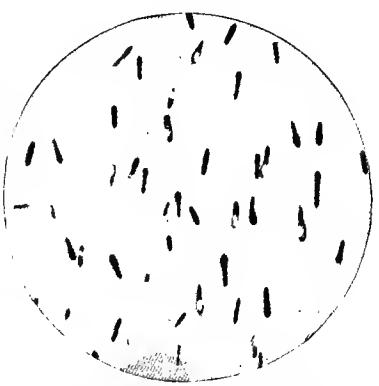
BACILLUS OF SYMPTOMATIC ANTHRAX.

Occurrence and Historical.—The disease known as symptomatic anthrax, black-leg, black-quarter, quarter-ill, Sarcophysema hæmorrhagicum bovis; "Kalter Brand, Rauschbrand" (German); charbon symptomatique, charbon bacterie (French), generally affects cattle and occasionally other animals, like sheep and hogs. It is due to a specific anaërobic gas-forming microörganism known as the *Bacillus* or *Clostridium sarcophysematos bovis,* bacillus of symptomatic anthrax or Bacillus chauveaui. The disease is particularly prevalent in mountainous countries, with deep and marshy valleys, also in flat countries, in which the pastures are exposed to periodical inundations. It is most prevalent during the hot season, and attacks particularly cattle between six months and two years old. The disease has undoubtedly been known for a long time, but it was formerly generally mistaken for anthrax. Bollinger (1875) and Feser (1876) first pointed out the difference between true anthrax and black-leg. They showed the specific bacilli in the emphysematous swellings and inoculated material containing them into ruminants and rabbits. Several French authors studied the organism in the following years, and Roux (1887) and Kitasato (1889) were the first to cultivate it artificially. The pathology and bacteriology of the disease were first more extensively investigated by Kitt, whose studies were made in the Bavarian Alps. The disease is quite prevalent in the United States, the majority of cases having been reported from Texas, Oklahoma, Kansas, Nebraska, Colorado, Indian Territory, and a number of Northwestern and Western States. According to Moore, infected localities have also been recently found in New York. Black-leg occurs in Germany, Austria-Hungary, Switzerland, and the other European countries. It has also been observed in Africa from Algiers to the Transvaal.

Pathologic Lesions.—The most important objective changes characterizing the disease are rapidly forming, rather diffuse, ill-defined swel ings of the skin and superficial muscles, which are first very firm and solid, but soon become infiltrated with air (emphysematous), so that they are crepitant to the touch and tympanitic upon percussion. To the palpitating finger these swellings impart a feeling as if there was paper beneath the skin. The most common seat of these emphysematous lesions is in the thick muscles of the hind- or fore-legs, from which fact the names black-leg and quarter-ill are derived.

The disease generally takes an acute, and, as a rule, fatal course. On postmortem examination the cadaver is generally much distended, not merely at the abdomen but over the entire external surface which is filled with gas. The formation of the latter continues after death. The subcutaneous tissue upon removal of the skin presents a gelatinous, yellowish or hemorrhagic appearance; the muscles appear dark, red brown or black brown. The soft, necrotic muscles crepitate and discharge gas on section. They are sometimes so full of air that they float on water like a piece of lung. The meat of animals dead from black-leg has a peculiar sweetish, rancid, non-fetid smell. Sometimes only one extremity shows these characteristic changes, at other times two, three, or all four are involved. While the parts affected by the disease always contain an enormous quantity of blood, the unaffected parts may be very pale and anemic.[1] In addition to these changes hemorrhages into the serous membranes and an accumulation of hemorrhagic serous fluid in the various body cavities are found. The liver and kidneys show evidences of parenchymatous degeneration; the former is sometimes filled with gas (foamy liver). The specific bacilli are found in the diseased muscles, serous hemorrhagic exudates, and gall-bladder, liver, etc. Many of the organisms are generally sporulating.

FIG. 135



Bacilli of symptomatic anthrax, showing spores. (After Zettnow.)

Morphology and Staining Properties.—The bacillus of emphysematous anthrax is from 2 to 6 micra long and 0.5 to 0.8 micron wide. It generally appears singly, or in pairs, rarely in long chains; some are cylindrical, others more oval (hence the name, clostridium), still others are club-shaped. French authors have compared the shape of the bacillus to snow-shoes. The clostridium shape is best seen after sporulation has taken place. The organism, unlike the anthrax bacillus, forms spores while in the body of the live animal. The spores are generally in the middle of the bacterium, sometimes more toward one end, and many of them, particularly in cultures, are found free. In young cultures the bacilli are quite actively motile and possess numerous flagella, which, however, break off easily and are generally difficult to demonstrate by staining methods. The bacillus stains in a peculiar manner with iodin solution. The vegetative

[1] A condition like this where an anemia of one part is brought about by a congestion in another part is called a *collateral anemia.*

form then appears blue, the clostridia black violet, and the spores remained unstained. In the bodies of sick cattle spore formation is generally well marked; the contrary is true in guinea-pigs infected experimentally. The bacillus keeps the stain when treated by Gram's method.

Cultural Properties.—The black-leg bacillus is a strictly anaërobic bacterium. According to Kitt the best method of obtaining pure cultures is the following: With a very fine funnel, gelatin and agar are poured into glass tubes 20 to 30 cm. long and 3 to 5 mm. wide, which furnishes a high narrow column of the medium. These glass tubes are fused at one end and closed at the other with a cotton plug. From four to ten of these *agar* and *gelatin tubes* are melted and cooled in the usual manner and then inoculated; the first one with a few drops of meat juice or blood from a case of black-leg, the others in the ordinary diluting manner, *i. e.*, No. 2 is inoculated from No. 1, No. 3 from No. 2, and so forth. In the last tube, No. 10, for example, there is so little of the original bacilli-containing material that a slow growth, with scanty colony formation, will occur. Whenever more abundant material is used in inoculation the growth in the incubator under anaërobic conditions is so rapid that no individual colonies are formed and the culture medium is broken up as a result of the extensive gas formation. In successful attempts with very high dilutions delicate, grayish, very finely granular, or punctate colonies from 0.5 to 1 mm. in diameter develop. They sometimes become larger, more compact, and less transparent, and attain the size of a millet seed. The growth never reaches the surface of the medium. In gelatin the colonies somewhat resemble frog's spawn; they liquefy the medium completely, except the uppermost zone, which is not invaded and remains solid. In the liquefied mass the growth sinks to the bottom and a whitish, rather scanty cloudy sediment collects. The addition of *cattle serum* to the gelatin is very favorable to the growth. In *nutrient bouillon* containing some sterile cattle blood serum the growth is abundant, the medium soon becomes cloudy, and gas is formed. A medium composed of bouillon (10 c.c.), to which 1 to 5 c.c. of fresh sterile blood obtained from the live animal has been added, enables the bacillus to grow in the presence of air. A good growth can also be obtained by placing a piece of fresh muscle (thorax muscle of a pigeon) procured in a sterile manner in the inoculated bouillon. The bacillus also grows in pure *sterile blood.* In this medium it forms spores abundantly and retains its virulency much better than in other media, in which it generally soon loses its pathogenic properties.

Animals Susceptible.—The bacillus is pathogenic to guinea-pigs, very slightly to rabbits, which, on the contrary, are very susceptible to the bacillus of malignant edema. In natural infection of cattle the bacilli gain entrance through wounds of the skin into which mud from marshy grounds has penetrated. This was demonstrated long

ago by Feser, who found bacilli like the black-leg bacillus in such mud and succeeded in producing the disease by inoculation into cattle and sheep. Cases of black-leg have been seen following castration. It is also held that the disease may be contracted by infected water or feed. Marek saw a case in a hog in which the infection had occurred through the tonsils. The black-leg bacilli appear to live and multiply as saprophytes in the soil. Direct transmission from one animal to another does not appear to occur. In infection through the intestines it is believed that spores enter the lymph and blood currents and are carried by them to some place in a muscle or in loose connective tissue, where aërobic bacteria have gained entrance through a wound. With these the black-leg bacillus, or its spores, enters into a symbiotic community, and the organism is now enabled to multiply and produce the disease.

Resistance.—The spores of the Bacillus sarcophysematos bovis are highly resistant. Sporulating bacilli contained in pieces of dry meat remain virulent for many years (10). The spores resist putrefactive processes for months, and they are believed to remain alive for a long time in manure. Spores contained in dried pulverized meat have been exposed in the steam sterilizer for five to six hours to a temperature of 100° C., and have still been found very virulent. Sheep have been killed by inoculating them with 0.1 to 0.2 gram of material treated in this manner. It requires fully seven hours in streaming steam of 100° C. to kill the spores in dried meat; dry heat of 104° C. has no effect after seven hours' action. Virulent spores have a *negative chemotactic* effect upon phagocytes, but avirulent spores freed of their toxins are taken up by phagocytes and destroyed. If toxin-free spores, however, are protected against phagocytosis by mechanical means (sand) or by chemical means (injection of lactic acid) they can still germinate and produce a fatal infection. Direct sunlight kills moist spores in eighteen hours; dried spores during the greatest summer heat in twenty-four hours. Corrosive sublimate has a comparatively strong disinfective power toward the spores; 1 to 500 solution kills spores from a culture in ten minutes; in moist fresh meat, in thirty minutes; in dried meat, after sixty minutes' exposure. The effect of carbolic acid is not very rapid; according to Kitasato, a 5 per cent. solution killed spores only after ten hours. The microörganism is a saprophytic soil bacterium, and its strong resistance makes the disinfection of infected marshy pastures practically impossible; they either must be abandoned or the animals having access to them must be protected by inoculation.

Vaccine Therapy and Protective Inoculation.—The first successful experiments in protective methods against emphysematous anthrax were undertaken by Arloing, Cornevin, and Thomas during the years 1880 to 1887. The first method used by French investigators consisted in the *intravenous injection* of 1 to 6 c.c. of expressed juice from infected muscles. This procedure confers immunity, but is very

dangerous because of the unavoidable risk of injecting a portion of the juice into the connective tissue, in which case a violent local infection generally leading to death results. Even when all the juice is injected into the veins, there still remains the danger of a fatal local infection at some point where an accidental hemorrhagic contusion in the subcutaneous or muscular tissue has occurred. This fact has given rise to the belief that emphysematous anthrax is often an intestinal infection, as already stated. Thomas' *vaccination method,* known as the "vaccination par le fil virulent," was the first to be practised more extensively. The procedure consists in saturating a bundle of silk threads with an attenuated culture of black-leg bacilli which have passed through the body of the frog. The silk threads are inserted in the tail by a special vaccination needle devised by Thomas. This method has been used in France on at least a million and a half head of cattle, with good results. Later, the French workers prepared two vaccines, one considerably attenuated, which was used for the first inoculation and one less attenuated for the second. Kitt's experiments on the resistance of the black-leg bacillus and its spores led him to work out what is now the most commonly used method in the protective inoculation of cattle against natural black-leg infection. Dried and ground-up muscles from cattle dead from emphysematous anthrax are exposed for five or six hours to steam of a temperature of 97° C., after which the powder is again completely dried. It was found that this powder in doses of 0.2 to 0.6 gram still killed sheep, but in smaller doses immunized and protected them. Kitt's method, used with excellent results in Europe, was somewhat modified in the United States by Noergaard, of the Bureau of Animal Industry, who exposed the ground-up infected muscles from cattle for six hours to 93° to 95° C. The latter vaccine has been successfully used on several million head of cattle. It may be remembered, however, that occasionally an animal treated with the vaccine dies from the inoculation, but this number is so small as to become a negligible quantity when compared with the great protective value of the vaccination.

Directions for the Use of Black-leg Vaccine.—The following is an abstract of the directions given by tbe Bureau of Animal Industry for the use of its prepared vaccine: The black-leg vaccine, as prepared by the Bureau, consists of a brownish powder, which is put up in packets containing either ten or twenty-five doses each. To prepare this powder in such a way that it may be injected hypodermically, it is necessary to use certain implements. The outfit consists of a porcelain mortar, with pestle, a small glass funnel, a measuring glass, and a syringe. For filtering the vaccine, absorbent cotton is most suitable. The syringe used has a capacity of 5 cubic centimeters, and the piston is graduated from one to five, each division being subdivided with half and quarter notches. The screw-regulator may be placed at any mark on the piston, thus insuring that the animal

to be vaccinated receives only the exact dose intended for it. The plunger is made of rubber; it should be air-tight in the glass barrel, and yet capable of being moved up and down smoothly. In order to prevent the plungers and washers from drying out, the small loose cap should be always tightly adjusted to the peg when the syringe is not in use. The hypodermic needles should be kept very sharp at the point, in order to pass easily through the skin, and when not in use should have a fine brass wire passed through each to prevent rusting on the inside. Whenever the point of the needle gets blunt it becomes very difficult to pass it through the skin, causing the fingers of the operator to become sore from attempting to force it through, and frequently the needle either bends or breaks. It is, therefore, of importance to have a small oilstone at hand on which to sharpen the point of the needle.

Before preparing the vaccine all the utensils, together with the syringe, must be sterilized thoroughly. This is done by putting the mortar, pestle, measuring glass, funnel, and needles in a pan of cold water and placing them over the fire. After boiling for ten minutes the pan with the contents should be allowed to cool off slowly; then remove the utensils from the water and wipe them dry with a clean linen cloth which has been previously boiled.

Preparation of the Vaccine.—Place the contents of one packet of the vaccine in a porcelain mortar and add a few drops of boiled water. Work the powder thoroughly with the pestle and then add, little by little, as many cubic centimeters of water as the packet contains doses. As the syringe contains exactly 5 c.c. it may be used for measuring the water. Place a small piece of absorbent cotton in the funnel and press it lightly into the upper end of the neck, sufficient to keep it in place; moisten the cotton with a few drops of boiled water and let it drip off. Stir the mixture in the mortar thoroughly, and before it has had time to settle, pour it into the funnel under which the measuring glass has been placed. The solution should not be perfectly clear. If this is the case, the cotton has been pressed too closely into the neck of the funnel.

Age of Animal and Dosage.—Calves, as a rule, should not be vaccinated until they are six months old. Under this age they are practically immune, and it has been claimed that when vaccinated before they are six months old they are likely to lose the artificial immunity induced and become susceptible again. Animals over two years old are rarely affected, and the mortality is so small as to make vaccination unprofitable. It is the animals between six months and two years old which should be vaccinated. Vaccination and castration should not be performed at the same time.

Ten days to two weeks should be allowed to elapse after vaccination before any surgical operation is undertaken, and if performed before the vaccination, ample time should be allowed for the part to heal and the animal to regain its lost strength. Animals one year

old or over are injected with a full dose of vaccine; that is, 1 c.c. of the solution. Under this age the dose may be reduced to one-half or three-fourths of a full dose, according to the size and development of the animal. Less than one-half dose should never be injected. In determining the dose for each animal, more consideration should be given to the size and development of the animal than to its exact age. The most convenient place for inoculation is on the side of the neck, just in front of the shoulder, where the skin is loose and rather thin. If the animals are secured in a dehorning chute, it is easier to vaccinate them on the side of the chest just behind the shoulder.

Steps in the Vaccination Process.—1. Sterilize outfit by boiling.

2. Place the contents of one packet in the mortar and add a few drops of water.

3. Work the mixture well with the pestle.

4. Add two to five syringefuls of water, according to the size of the packet, and stir well.

5. Place cotton in glass funnel and moisten with water.

6. Filter vaccine into the glass or bottle.

7. Secure the animal to be injected.

8. Insert the needle through the skin.

9. Fill the syringe and adjust the screw regulator on the piston. If the first animal is a yearling or older, place regulator No. 1 on the syringe.

10. Fit the peg of the syringe into the cap of the needle and inject the dose.

11. Withdraw syringe and needle together. If the syringe is removed from the needle before the latter has been drawn out of the skin some of the injected vaccine will flow back through the needle and be lost. In this case the animal does not get a full dose, and will consequently be insufficiently protected.

Black-leg Toxins and Antitoxins.—If cultures of the bacillus of emphysematous anthrax in fluid culture media are filtered through a Pasteur-Chamberland or Berkefeld filter, soluble toxins cannot be demonstrated in the filtrate. Grassberger and Schattenfroh, however, have devised a somewhat complicated method of growing the organism in particular media and filtering its cultures through powdered chalk in such a manner that a germ-free filtrate containing a powerful toxin is obtained. The properties of the latter are described as follows: The toxin seems to be pathogenic for all warm-blooded animals; its effect seems to be the same, calculated per body weight of animal in the case of cattle, sheep, guinea-pigs, rabbits, monkeys, dogs, hedgehogs, mice, chickens, and pigeons. Cold-blooded animals are not susceptible. After injection of a single fatal dose into a guinea-pig, a painful, doughy or elastic swelling, which spreads after eight to ten hours, is formed at the place of injection. The skin early shows hemorrhages. There is first a slight elevation of temperature, which later becomes subnormal.

The animal becomes restless and dies within two to four days, a hemorrhagic discharge from the mouth and nostrils appearing shortly before death. The postmortem examination shows a hemorrhagic œdema of the subcutaneous tissue and hemorrhagic serous exudates in the body cavities. The two authors named have succeeded in obtaining germ-free solutions, of which two milligrams or even one-half milligram would kill a guinea-pig of one-half pound weight (250 grams). The *toxin* which has these pathogenic properties is not very stable; it is destroyed in one hour if heated to 50° C., 1 per cent. carbolic acid solution destroys it in twenty-four hours, but chloroform has no deleterious effect upon it, and is used as a means for its conservation. The authors also succeeded in preparing an antitoxin. Guinea-pigs could not be actively immunized against the toxin, on the contrary, a repeated injection of small doses produced a hypersusceptibility. Rabbits, however, can be actively immunized, and also cattle. The blood serum of cattle after a systematic course of hyperimmunization lasting four to five months often develops a high antitoxic or immunizing value, and the antitoxin has the great advantage of being apparently quite stable when properly kept and stored. It was found to be thermostabile, and heating it for three hours at 60° C. had no detrimental effect upon it. The "Rauschbrand" antitoxin of Grassberger and Schattenfroh, however, is valueless in the treatment of natural or artificial infection with the black-leg bacillus, because death is not caused by the soluble toxin but by factors which are in no way affected by the antitoxin. The latter, particularly on account of its very peculiar behavior toward its toxin, is, therefore, of theoretical interest only.

QUESTIONS.

1. What are the other names of the disease black-leg?
2. What is the cause of this disease of cattle?
3. At what age are cattle most susceptible to the disease?
4. Describe the most characteristic pathologic lesions of symptomatic anthrax.
5. What is the meaning of the terms emphysematous, crepitant, tympanitic?
6. What is a collateral anemia? Explain its occurrence in black-leg.
7. Describe the morphology and staining properties of the Bacillus sarcophysematos bovis.
8. What is a clostridium?
9. Describe the cultural properties of the bacillus of black-leg.
10. What substance added to the ordinary culture media greatly favors the development of the organism?
11. Describe Kitt's method of cultivating the bacillus from the juice of infected meat or from a small piece of the latter.
12. Describe the differences in the action of the anthrax bacillus and the Bacillus sarcophysematos bovis in the blood of animals dying from these two diseases, respectively.
13. What animal may be used to differentiate by inoculation experiments between the black-leg bacillus and that of malignant edema?
14. What is the natural mode of infection of symptomatic anthrax in cattle?
15. Discuss the resistance of the spores of the bacillus.
16. Which spores are and which are not destroyed by phagocytosis? How can the phagocytosis of toxin free spores be prevented?

17. How can pastures infected with the black-leg bacillus and its spores be disinfected?

18. Describe the first experiments in protective inoculation against "Rauschbrand" infection.

19. What are the dangers of the first method employed by Arloing, Cornevin, and Thomas?

20. What is Thomas' method of vaccination?

21. What is Kitt's method? What is the method generally employed in the United States?

22. Describe in detail the steps in the use of black-leg vaccine prepared by the Bureau of Animal Industry.

23. What kind of animals should be inoculated; what kind should not?

24. Does the bacillus of symptomatic anthrax form a soluble toxin? how can it be obtained? what are its properties?

25. Can an immune serum against such a toxin be prepared? What are its effects in the prevention and cure of the natural disease?

CHAPTER XXII.

THE BACILLUS OF MALIGNANT EDEMA AND SIMILAR BACTERIA —BACILLUS OF GASTROMYCOSIS OVIS—BACILLUS AËROGENES CAPSULATUS.

BACILLUS OF MALIGNANT EDEMA.

Occurrence and Historical.—A spore-forming anaërobic bacterium, causing a peculiar form of wound infection and inflammation, frequently occurs in soil, sewage, dust, grasses, the intestinal contents of animals, manure, and putrefying animal substances. Feser, in 1876, working with "Rauschbrand," evidently saw these bacilli, but they were first more exactly recognized by Pasteur, who was able to produce in guinea-pigs and rabbits by the inoculation of putrid material a disease characterized by an edematous inflammation at the place of inoculation. Pasteur named the bacterium which produced these changes "Vibrion septique." Robert Koch, in 1881, showed that this disease was not a true septicemia, and he called it malignant edema and the microörganism the bacillus of malignant edema. Its pathogenic significance was later studied by a number of observers, including Brieger and Ehrlich, Jensen and Sand, Kitt, and others. It has been found to be the cause of wound complications in man and domestic animals, among the latter particularly in horses, cattle, and sheep, very rarely in hogs, dogs, and cats.

Pathologic Lesions.—At the place of infection with the bacillus of malignant edema the tissues are swollen and considerably infiltrated with a yellowish or reddish serous exudate. The fluid contains numerous gas-bubbles. Hemorrhages are found here and there in the tissues. The peritoneal cavity contains a moderate amount of reddish serous fluid; the peritoneum is congested, dull, but does not show any fibrinous deposit. In cases where malignant edema follows delivery the uterus is found to be flaccid and insufficiently contracted; the wall of the uterus and the pelvic connective tissue are edematous. The cotyledons, according to Carl, are changed into a mushy, dirty, ill-smelling mass. The spleen, as a rule, is not much changed, but it may be swollen, edematous, and emphysematous. The liver shows parenchymatous degeneration, the lymph nodes are swollen, the lungs are edematous and congested, and the heart muscle is soft and flabby; the blood, as in anthrax, does not promptly and firmly coagulate, and putrefactive changes set in early. If juice from an emphysematous organ or location is examined

microscopically long, slender rods are seen; often also pseudofilaments. The bacilli somewhat resemble anthrax bacilli, but have rounded and sometimes even pointed ends, instead of square ends.

Morphology and Staining Properties.—The bacillus of malignant edema varies much in length; its average is from 2 to 4 micra; some individual bacteria are much longer, and the pseudofilaments may have a length of 15 micra. The diameter of the rods and pseudofilaments is generally 1 micron. Spores are found in moderate numbers in the serous exudates; they are very numerous in cultures. They are, however, only found in the single bacilli, not in the filaments; they are oval, generally situated in the middle, occasionally toward the end of the rods; they bulge out the bacillus somewhat, but not to such an extent as the symptomatic anthrax bacilli. Jensen found that some stems of malignant edema bacilli form pseudofilaments either in the cultures or in bodies of animals. The bacillus is motile and possesses numerous flagella. The organism stains with the ordinary watery anilin stains and keeps Gram's stain if the decolorization in alcohol is not continued very long.

FIG. 136

Bacillus of malignant edema. (Park.)

Cultural Properties.—The organism is strictly anaërobic. It grows at room temperature and in the incubator in the absence of oxygen, on all the ordinary media, particularly well in the presence of a salt of formic acid or glucose. In *gelatin stick* cultures, small, white, shining round colonies are formed along the stab. As these increase in size gas-bubbles appear and the medium becomes liquefied and is changed into a grayish-white cloudy fluid. *Agar* becomes cleft and torn in consequence of the gas formation during the growth. *Bouillon* becomes cloudy and forms a whitish sediment after two to three days; the upper strata then clear up and small gas-bubbles continue to rise to the surface. The bacillus grows well on coagulated blood serum. On *potatoes* it multiplies likewise, but the growth which it forms is invisible. When growing in blood serum the bacillus produces a very fetid smell, due to the putrid decomposition of the serum albumin.

Resistance.—The spores of the bacillus of malignant edema are very resistant, and in this respect act much like the spores of the Bacillus sarcophysematos bovis. Sunlight appears to have little effect upon them, likewise 5 per cent. carbolic acid.

Natural Infection.—This generally takes place from cutaneous wounds, from the denuded surface of the parturient uterus, etc. Infections in man and the horse have been seen following medicinal injections with unclean hypodermic syringes. The mucous membranes of the mouth and pharynx also form a portal of entrance. It is believed that the organism or its spores may enter the circulation

from the intestines and may be carried to a contused place which furnishes a favorable location for multiplication. Kitt has reported cases in which it appears that sheep may contract a malignant edema of the lungs by inhalation.

In natural infection the bacilli are found in the serous or watery exudates. They occur in enormous numbers in the connective tissue of the affected part, in more moderate numbers in the superficial portions of the muscles and very scantily in its deeper substance.

Artificial Inoculation.—Equines, guinea-pigs, rabbits, rodents, cattle, chickens, and pigeons are susceptible to artificial inoculation; dogs and cats only very slightly.

Protective Inoculation.—Leclainche and Valee have shown that animals may be protected against malignant edema infection by inoculation of spore-containing material which has been heated for seven hours at 92° C. Animals repeatedly treated with such vaccines also develop an immune serum which will produce passive immunity in other animals. All these tests are still in an experimental stage, and neither protective virus inoculation nor passive immunization has ever been used in practice.

BACILLUS OF GASTROMYCOSIS OVIS.

Occurrence.—In the northern parts of Europe, Iceland, Norway, the Faroe Islands, Scotland, Denmark, and also in North Germany, a disease of sheep occurs which is known as bradsot, braasod, bradapestina, bradasottina, bradafarid, braxy, or technically, as gastromycosis ovis. The disease runs a very rapid course. A sheep apparently well in the evening may be dead the following morning. The disease was described as early as about the middle of the eighteenth century. The first bacteriologic examinations were made by Kingberg, and the first description of the bacillus of bradsot was published by Nielsen, whose findings were confirmed and extended by Jensen.

Pathologic Changes.—Jensen describes these as follows: The mucosa of the stomach and small intestine exhibits a serous hemorrhagic infiltration, likewise the abdominal connective tissue, which may also show infiltration with gas. The mucosa of the "lab" stomach (rennet) is sometimes necrotic, and upon microscopic examination the tissues here show enormous numbers of bradsot bacilli. When the disease is produced by the experimental subcutaneous inoculation the animals develop hemorrhagic infiltrations of the deeper muscles, often with gas infiltration, and the pathological picture very much resembles symptomatic anthrax. Jensen failed to produce bradsot in sheep by feeding them with hard material, including thistles which had been contaminated with cultures of the bacilli.

Morphology.—The bacillus of gastromycosis ovis is a large rod, 2 to 6 micra long, 1 micron wide. It has rounded ends, and is found

singly in the serous cavities of sheep dead from the disease, and frequently in longer chains and pseudofilaments in the interior of the parenchymatous organs. It forms spores in the body of the infected animal, and also rapidly in artificial cultures. The spores are generally in the middle, more rarely toward one end of the bacillus. The organism is motile, and possesses flagella.

Cultural Properties.—The bacillus, under anaërobic conditions, grows in the ordinary culture media; but a more abundant growth develops only upon the addition of a moderate amount of glucose, which is fermented with gas formation. It grows well in *milk*, which it coagulates in consequence of abundant acid formation. The coagulated casein is not peptonized. The organism does not grow in a medium of acid reaction, and when it has, in its growth in an alkaline medium, formed a certain amount of acid from glucose or lactose, the development ceases. It is very probable that the bacillus occurs in the upper strata of the soil, but this has not been definitely established.

OTHER ANAËROBIC GAS-FORMING BACILLI.

Kitt discusses a number of affections in animals under the name of "pseudo-Rauschbrand." These are either clearly due to the bacillus of malignant edema or one of its varieties or organisms more nearly related to the Bacillus sarcophysematos bovis. According to Kitt and to Carl the disease of cattle called in German "Geburts-rauschbrand" (parturient emphysema) is due to a typical bacillus of malignant edema. This bacterium has also been found by Jensen, Horne, Hutyra, and Kitt as the caue of the same disease in horses. The Bacillus œdematis thermophilus, found by Kerr and Novy in a cow, is probably a variety of the Bacillus sarcophysematos bovis, from which it differs in certain minor cultural features, and in the fact that it is very pathogenic for rabbits and rats, which are relatively resistant to the typical bacillus of symptomatic anthrax.

A peculiarly interesting member of the group is a bacillus pathogenic for whales described by Nielsen. For centuries the inhabitants of northern Norway have caught whales in a unique manner. They noticed that these animals sometimes suffered from emphysematous inflammations of the muscles, and they poison arrows by dipping them into the juices of such diseased meat. If whales are hit by such poisoned arrows they sicken rapidly (within eighteen to thirty-six hours) and can be easily caught. Nielsen, who examined the emphysematous whale meat and the poisoned arrows, found an organism of the Bacillus sarcophysematos bovis type in enormous numbers. The muscles of the whale infected with the bacillus of Nielsen show the same anatomical changes as the meat of cattle sick with emphysematous anthrax.

Reindeer Plague.—Among the reindeer of Lapland an occasionally very epidemic disease which kills thousands of calves and young

animals has been observed. The cadavers show emphysematous edema over various parts of the body. Hemorrhages from the nose generally precede the fatal termination. Lungdren and Bergman found a bacterium much like the symptomatic anthrax bacillus in the edematous fluid and serous exudates of the sick and dead animals. The bacillus of reindeer plague grows best aërobically; it is 1.6 to 4.8 micra long, 0.7 to 0.8 micron wide; motile, with flagella; it forms oval spores in the centre or at one end; occurs singly, in pairs or in chains and pseudofilaments, and stains with the watery anilin stains and by Gram's method. It grows between 12° and 38° C. The spores are very resistant, and the organism is pathogenic in experimental inoculation to reindeer, sheep, guinea-pigs, white mice, chickens, pigeons, and sparrows.

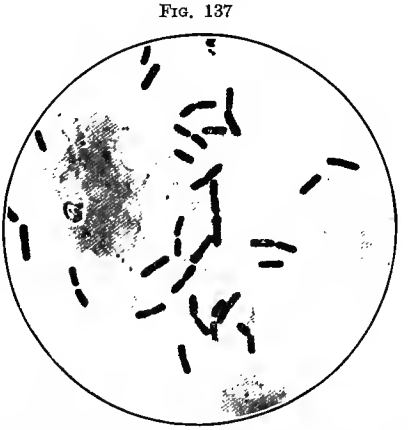
Other gas-forming, edema-producing bacilli which have been described, but which are not strictly anaërobic, are the following:

Novy's Bacillus œdematis maligni II, Klein's Bacillus œdematis sporogenes, and *Sanfelice's Bacillus œdematis aërogenes.*

BACILLUS AËROGENES CAPSULATUS (WELCH), OR BACILLUS EMPHYSEMATOSUS (FRAENKEL).

This bacillus, described by Welch, Fraenkel, Nuttall, Flexner, Howard, Jr., and others, is an anaërobic gas-forming sporogenous microörganism, and has been found a number of times as the cause of terminal infections in man. It belongs to the group of malignant

FIG. 137



Bacillus aërogenes cupsulatus infecting a human liver. × 1000. (Author's preparation.)

edema and symptomatic anthrax bacilli, but has never been encountered as a cause of natural infection in animals. It has recently been found by MacNeal, Latzer, and Kerr as a normal inhabitant of the intestines of healthy persons, and it is probably also found in

the soil. The bacillus is from 3 to 5 micra long and about 1 micron in diameter; it generally occurs in pairs and irregular groups, rarely if ever in chains. It has rounded ends, is not motile, possesses no flagella, stains by the ordinary methods, and keeps Gram's stain. It forms spores but not in the infected human body, only in artificial culture media, best on Loeffler's blood-serum mixture. The vegetative forms of the organism are not very resistant; the spores are, however, quite resistant. In experimental work the bacillus has been shown to be pathogenic to rabbits, guinea-pigs, and pigeons.

QUESTIONS.

1. What is the bacillus of malignant edema? Where is it found?
2. What relation has the vibrion septique to the Bacillus œdema maligni?
3. What beings are susceptible to infection with this bacillus?
4. Describe its most characteristic pathologic lesions.
5. What is the character of the blood after death due to infection with this bacterium?
6. Describe the morphology of the bacillus. In what morphologic features does it differ from the anthrax and from the symptomatic anthrax bacilli?
7. Describe the spores of the malignant edema bacillus.
8. Describe its cultural properties.
9. Discuss its resistance.
10. How does natural infection take place?
11. What animals are susceptible?
12. What work has been done as to protective inoculation against malignant edema?
13. What is bradsot, or gastromycosis ovis? Where does it occur?
14. Describe the pathologic changes of the disease.
15. Describe the bradsot bacillus.
16. What is meant by pseudo-Rauschbrand bacilli?
17. What is parturient emphysema due to?
18. What is the Bacillus œdematis thermophilus?
19. Describe the Nielsen gas bacillus of whales.
20. What is reindeer plague? What bacillus causes it? Describe its morphology, cultural properties, and pathogenesis.
21. Name some edema-producing aërobic gas bacilli.
22. What is the Bacillus aërogenes capsulatus? What human and what animal diseases does it cause?
23. Where is it found?

CHAPTER XXIII.

BACILLUS OF TETANUS.

Occurrence and Animals Susceptible.—Tetanus, or lockjaw, is a disease of man and some of the lower animals, which has been known for a long time. It is, in fact, mentioned by Hippocrates, and it had been noticed that it frequently follows lacerated, deep-seated contaminated wounds. The disease is characterized by clonic and tonic convulsions of the voluntary muscles, but it has no characteristic anatomic or histopathologic lesions, and a diagnosis of tetanus cannot be made from a postmortem examination unless it is combined with a bacteriologic examination, including animal inoculation experiments. The clinical symptoms, however, are so characteristic that it is easy to diagnosticate a case of typical tetanus in man or animals. The disease, as a rule, follows wound infection; the wound so infected may be the umbilicus of the newborn or the uterus after parturition. Man and the horse are most susceptible to natural infection, but cattle, sheep, and hogs are likewise subject to tetanus. The dog is only slightly susceptible, likewise the cat. Of experimental animals, mice, guinea-pigs, and rabbits are susceptible. Tetanus may develop in exceptional cases as a cryptogenetic infection, *i. e.*, one of hidden, secret origin, from the intestinal tract; this manner of origin, however, does not occur in the horse.

Historical.—The first investigator to succeed in producing artificial experimental tetanus was Nicolaier in 1885. He inoculated mice, rabbits, guinea-pigs, and dogs with garden earth, and by this means produced tetanus in the three former animals but not in the dog. At the point of inoculation he found slender bacilli, but in his experiments he failed to obtain them in pure cultures. Rosenbach, in 1887, saw identical bacilli in man in a gangrenous wound which had led to the development of lockjaw. Finally, in 1889, Kitasato succeeded in obtaining the tetanus bacillus in pure culture by raising it under strictly anaërobic conditions.

Morphology and Staining Properties.—The tetanus bacillus when obtained from a gelatin culture is a slender rod 2 to 4 micra long, 0.3 to 0.5 micron wide. It has slightly rounded ends. In addition to individual bacilli, chains of several rods, forming slender filaments, are found; the older the culture the more numerous are the latter. In older cultures raised on gelatin at room temperature many spore-bearing bacilli are seen. Young tetanus bacilli show a slight motility, which can be best demonstrated on a warm stage. The bacilli

possess numerous peritrichous flagella, estimated from thirty to fifty and even up to one hundred. Spore formation in cultures kept anaërobically in the incubator occurs after twenty-four to thirty hours; at room temperature in gelatin tubes after eight to ten days. The most abundant sporulation occurs in sugar free bouillon and on blood serum. The tetanus bacillus forms its spore at one end, it is round and much larger in diameter than the vegetative form of the bacillus, namely, 1 to 1.5 micra. A sporulating bacillus somewhat resembles a drum stick and for this reason, the microörganism is also called the *drum stick bacillus of tetanus*. When the culture medium contains sugar or glycerin the spores are sometimes oval and not perfectly spherical.

The tetanus bacillus stains with the ordinary watery anilin stains and is Gram positive. The spores and the flagella can only be stained by special methods.

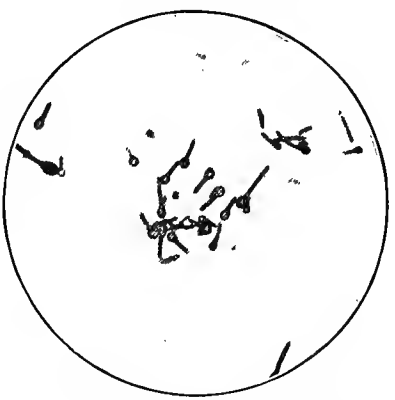
Anaërobic Methods.—The bacillus is anaërobic and does not grow in the presence of oxygen. A variety of methods are used to exclude the atmospheric air. The cultures may be kept in an air-tight jar in which the common air has been replaced by hydrogen or in a jar in which the oxygen has been absorbed by pyrogallic acid and caustic soda solution. The bacillus may also be raised in a bouillon from which the air has been expelled by prolonged recent boiling and the surface of which has been covered by oil or butter, or in gelatin stick cultures, which after inoculation are covered by a high layer of gelatin containing some glucose.

Cultural Properties.—On *gelatin plates* kept at 20° C. (room temperature) colonies of the tetanus bacillus become visible on the third day. The small young colonies under a low magnification show a central solid area surrounded by radiating cords, bands or filaments. The young colonies somewhat resemble those of the Bacillus proteus; at other times they appear more like those of the Bacillus subtilis. In a *gelatin stick culture* the growth begins about one-half inch below the surface and proceeds downward, forming at the same time lateral projections which appear more or less cloudy. After the tenth day the gelatin becomes more and more liquefied, and simultaneously a very fetid gas is formed. On agar the growth is similar but there is no liquefaction. In *bouillon* kept at 39° C. there is a very rapid growth with marked clouding and the appearance of fine gas bubbles. Sedimentation begins to show on the fifteenth day. The gases formed are carbon dioxide and ethane and methane gas. During their formation the alkalinity of the fluid increases, provided no sugar is present. The tetanus bacillus does not grow at temperatures below 14° C.; at 18 to 20° C. the growth is slow and easily visible to the naked eye only after one week; at 20° to 25° C. a good growth develops in three to four days; the organism grows best at 36° to 39° C.

Tetanus Bacilli and Aërobic Bacteria.—The tetanus bacillus when grown in artificial pure cultures is, as has been stated, strictly anaërobic

and provision must be made to exclude the oxygen of the air. In soil, manure, and wounds, however, it can grow even without the careful exclusion of oxygen when it is associated with aërobic micro-organisms, with which it may enter into a symbiotic union. The aërobic bacteria consume the oxygen, and in this way enable the tetanus bacillus to multiply. On this account the most dangerous wounds are those which from the beginning have been contaminated with both tetanus and other bacteria. If such wounds are thoroughly cleansed with antiseptics which destroy the other bacteria, but not the tetanus spores, lockjaw may yet not develop in spite of the presence of these spores, which do not readily germinate and multiply when alone. It is also important to note that tetanus spores which have been freed from the tetanus toxin by washing with antiseptics may be taken up and destroyed by phagocytes. It is claimed that tetanus bacilli can be successively so changed that they will grow in the presence of oxygen, losing, then, their toxicity and pathogenic character. This statement, however, is doubted by some investigators, who believe that these aërobic, non-toxic bacilli are from the beginning a pseudotetanus bacterium.

FIG. 138



Bacillus tetani, spore formation. (Author's preparation.)

Resistance of Spores.—The spores are exceedingly resistant to heat and antiseptics, even more so in some respects than anthrax spores. They can survive complete drying out for many years; if raised primarily under the most favorable conditions, they can, according to Smith's experiments, withstand exposure to steam of 100° C. for one hour, and it is not until after an exposure of seventy minutes that all tetanus spores are safely killed.

The Bacillus as a Saprophyte.—The tetanus bacillus exists extensively in the outside world, particularly in garden earth and where there has been much manuring; in warm countries, however, it is present independent of any manuring. Some investigators believe that the tetanus bacillus exists in the ground only where the latter has come in contact with the feces of horses and other herbivorous animals which harbor the organism in their intestinal tract; it is, however, more generally held that it occurs in the soil independent of admixture with fecal matter.

Tetanus in the Horse and Man.—In horses tetanus is very common after nail wounds of the hoofs and after castration; in man after

deep puncture wounds or ragged wounds of the hands (Fourth of July injuries). In both man and the horse natural tetanus infection almost invariably leads first to spasms in certain groups of muscles and then progresses symmetrically to the other muscles of the body. The period of incubation varies in the horse from four to five days up to three weeks. According to Behring, one of the earliest and most important symptoms occurs in the membrana nictitans of the eye, which, when the head of a horse sick with tetanus is raised, is found to cover about one-half the eyeball and to spread as the disease progresses. The head and neck of the animal are elevated until the upper border of the neck describes a straight line; in extreme cases it is even concave, and forms what is called a deer neck. Mastication is difficult in consequence of the trismus of the muscles of mastication, and finally becomes impossible. The nostrils are dilated. The tail in consequence of the contraction of its extensor muscles is stiffly elevated. Finally the spinal column is curved (in opisthotonos position), and the muscles of the neck and thorax become stiff and very hard. All reflexes are accentuated, and a slight irritation will bring about convulsions. Death results from progressive dyspnea. Cattle and sheep also suffer from tetanus, but not nearly as frequently as horses.

Tetanus in Laboratory Animals.—The picture of tetanus is different when small laboratory animals, such as guinea-pigs and rabbits, are inoculated subcutaneously or intramuscularly. In this case the contractions begin in the group of muscles located nearest the point of injection. If the inoculation, for example, has been made into one of the hind legs it is the first to be affected, and in succession the other hind leg, the front legs, and, finally, the muscles of the back become involved. Increase of reflex irritability generally does not occur, but if it does it is observed only shortly before death. It has been noticed that tetanus bacilli when inoculated experimentally do not multiply at the place of inoculation; on the contrary, soon decrease in numbers. The disease is produced by the absorption of the toxins of the bacillus. Tetanus is one of the best examples of a pure toxemia.

Tetanus Toxin.—Tetanus toxin, like that of diphtheria, is soluble; it easily goes into solution if the bacilli are raised in a fluid culture medium. They must, however, be kept under strictly anaërobic conditions, because only under these will they produce a large amount of toxins. If air is present the bouillon will be of low toxic value. An ordinary slightly neutral bouillon containing 1 per cent. of peptone and 0.5 per cent. of sodium chloride is a good medium, but it must contain neither glycerin nor sugar, because the resulting acid formation will interfere with the toxin production. As the latter does not proceed very abundantly during the first few days of growth the cultures must be kept under anaërobic conditions in the incubator for ten or more days. The bacteria are removed from the bouillon by filtering it through a Pasteur-Chamberland filter

which has been carefully tested. The filtrate so obtained is free from bacteria and spores, and contains the tetanus toxin in solution. A good filtrate should contain a sufficient quantity of a strong toxin, so that one two-hundred-thousandth part of a cubic centimeter will kill a mouse of about 10 grams' body weight. Some investigators have even, by special means, obtained a filtrate five times as strong, of which the one-millionth part of a cubic centimeter would kill a mouse of about 10 grams' body weight. By saturating the filtrate with ammonium sulphate, Brieger, Fränkel, Buchner, and others succeeded in precipitating the toxin in the form of a powder, of which the one ten-millionth part of a gram would kill a mouse of 10 grams' body weight. Other animals, however, are more susceptible than the mouse to the fatal action of tetanus poison; the horse, for example, is twelve times as susceptible, and on the basis of the above figures a horse weighing 2000 pounds can be killed by one twelve-hundreth gram, or about one-eightieth of a grain of dried tetanus poison. Such powerfully poisonous effects are almost inconceivable. The tetanus toxin is even more powerful than the venom of the most dangerous snakes, such as the cobra. Man also is more susceptible than the mouse, but probably less than the horse, which is the most susceptible animal. The exact susceptibility of man as compared with the mouse and the horse is not known, since it cannot, of course, be ascertained by experimental work. The guinea-pig is 6 times as susceptible as the mouse; all other animals tested are less susceptible than the mouse; the rabbit 150 times less susceptible, the goose 1000 times, the pigeon 4000 times, and the chicken 30,000 times. The latter figure indicates that for each gram or pound of body weight of chicken 360,000 times as much tetanus toxin is needed as for each gram or pound of body weight of a horse in order to produce the same fatal effect.

Period of Incubation of the Toxin.—Bacteria and their toxins never act like purely chemical poisons, such as acids, alkalies, or salts (strychnine or hydrocyanic acid and its salts, cyanide of potash). Such poisons may be given in doses large enough to produce death almost instantly. However, if the most pathogenic bacteria or their toxins are inoculated into an animal even in very large doses, death never takes place immediately; a certain period of time always elapses between the inoculation and the first symptoms. When tetanus toxin is inoculated into a mouse or guinea-pig the period of incubation becomes shorter as the dose is increased; but it is impossible to go beyond a certain minimum period of incubation, no matter how much the toxic dose is increased. If mice receive 3600 times the fatal dose of tetanus toxin the period of incubation is shortened to a minimum of eight hours.

Chemistry of the Toxin.—Instability.—The chemistry of the tetanus toxin is absolutely unknown, and it can only be identified by its effect upon experimental animals; but it can, of course, also

be estimated quantitatively by ascertaining the minimum fatal dose of a filtrate containing the tetanus toxin. It must be understood, however, that the toxin is very unstable in the watery solution represented by the filtrate; it soon loses in intensity, and is easily damaged by heat and chemicals and antiseptics. The exposure of a filtrate containing tetanus toxin to a temperature of 65° C. for five minutes or of 60° C. for twenty minutes will almost completely destroy the toxin. Electric currents passing through a filtrate will destroy the toxin in a few hours. Mineral acids and alkalies destroy it even in very weak concentration; organic acids require stronger concentration. The poison can easily be attenuated by the addition of certain antiseptics. Iodide trichloride (ICl_3), if present to the slight degree of one one-hundredth per cent. will in one hour greatly attenuate the toxin.

Action of the Toxin upon the Nervous System.—The effect of the tetanus toxin upon the animal body is not yet well understood. It is, however, very probable that the poison acts upon the central nervous system by uniting with the cellular elements forming it, and, further, that the toxin travels from its first place of deposit in the connective or intramuscular tissue along the axis cylinders of the peripheral nerves toward the central nervous system. Wassermann has shown that if tetanus toxin is mixed with ground-up guinea-pig's brain the toxin becomes united with the brain substance, making it evident that an affinity exists which binds the tetanus toxin to the tissue of the central nervous system.

Component Bodies.—Tetanus toxin, as it is present in the filtrate, is composed of two bodies, tetanospasmin, a substance which has an affinity for and which affects the central nervous system and causes the convulsions, and tetanolysin, which has the property of dissolving red blood corpuscles.

Antitoxin Formation.—When tetanus toxin is injected into an animal an antitoxin is produced. The exact location where the latter is formed, whether in the central nervous system or not, is unknown; but the antitoxin is contained in the blood serum, and it can be obtained by allowing the blood to coagulate so that the serum can be separated from the clot or coagulum. Tetanus antitoxin is manufactured in the blood serum of the horse. The general principles and steps in forming and procuring it are as follows:

1. Inoculate a veal bouillon, containing 1 per cent. peptone and 0.5 per cent. sodium chloride, neutralized with carbonate of magnesium, and then slightly acidulated with 0.1 per cent. lactic acid from a pure culture of tetanus bacilli. Keep under strictly anaërobic conditions in the incubator for ten or more days.

2. Filter through an unglazed porcelain filter (Pasteur-Chamberland).

3. Ascertain the minimum dose which will kill a mouse of about 10 grams within four days.

4. Into a healthy horse (previously tested with mallein and tuberculin) inject either (*a*) a very small dose of strong tetanus toxin (this method is now little used); (*b*) a small dose of an attenuated toxin (attenuated with ICl_3, iodide trichloride); (*c*) a toxin-antitoxin mixture which is not completely neutralized, but contains a small excess of toxin; (*d*) first antitoxin and after eighteen to twenty-four hours, toxin.

5. Repeat injections of toxin at intervals of a few days and increase the doses.

6. Draw a small amount of blood from the jugular vein and test the antitoxic value of the serum on guinea-pigs and mice.

7. After two to three months or longer, when the antitoxic value of the serum is found to be high, draw off (ten to fourteen days after last injection) several thousand cubic centimeters of blood under the strictest aseptic precautions. Collect it in sterile vessels and allow it to coagulate in the refrigerator. Separate the serum from the clot and add, as a preservative, 0.5 per cent. carbolic acid or 0.4 per cent. tricresol. Distribute into small dark bottles and keep in a cool, dark place.

Dosage.—A good tetanus antitoxin is used in the following doses: Immunizing or preventive dose—10 to 20 c.c. for a horse; for a smaller animal, 5 to 10 c.c. The passive immunity conferred upon an animal is soon lost. Hence, when a wound in a horse, made accidentally or by operative procedure, does not heal very promptly, it is well to give a second immunizing injection ten days after the first. A unit of tetanus antitoxin is approximately ten times as large as a unit of diphtheria antitoxin.[1]

One unit of tetanus antitoxin is defined as the amount of antitoxin required to neutralize the effects of 1000 times the minimum dose of tetanus toxin fatal to a guinea-pig of 350 grams. If the guinea-pig is protected from death during the first four days after the injection of the toxin-antitoxin mixture, which must have been prepared fifteen minutes before the injection, the dose of antitoxin is called one unit of tetanus antitoxin.

The United States Government has adopted this unit of tetanus antitoxin and provides those who manufacture antitoxin with a standardized strong tetanus toxin. The German standard unit of tetanus antitoxin is that amount which will neutralize the amount of a standard toxin necessary to destroy 40,000,000 grams of mouse. The French standard is expressed by indicating the weight of antitoxic serum necessary to protect one gram of mouse against a minimum fatal dose of a standard strong toxin. If one-thousandth of a gram (0.001) is necessary to protect a mouse weighing 10 grams, one ten-thousandth of a gram will protect one gram of mouse weight; hence, such an antitoxic serum would be called a 1 to 10,000 antitoxic serum.

[1] One unit of diphtheria antitoxin is that dose which will protect a guinea-pig of 250 grams against the subcutaneous injection of 100 times the minimum fatal dose of a strong, fresh, diphtheria toxin, so that the guinea-pig lives at least four days, but will die after four days.

QUESTIONS.

1. What are the characteristic pathologic lesions of tetanus? What are the most characteristic clinical manifestations of the disease?
2. What animals are most susceptible to natural tetanus infection?
3. What is a cryptogenetic infection?
4. Who discovered the tetanus bacillus? who first grew it in pure culture?
5. Describe the morphology of the tetanus bacillus.
6. What conditions favor spore formation?
7. What methods may be used in raising tetanus cultures under anaërobic conditions?
8. Describe the cultural properties of the Bacillus tetani.
9. Discuss the resistance of the spores of the microörganism.
10. Where is the bacillus found as a saprophyte?
11. Under what conditions does tetanus frequently make its appearance in horses and human beings?
12. Describe the early symptoms of tetanus in a horse.
13. Describe the formation and properties of the tetanus toxin.
14. Describe the process of separating the bacilli and their spores from the toxin in order to obtain a germ-free solution of the latter.
15. Discuss the susceptibility of various animals toward the tetanus toxin; what animal is most susceptible to its poisonous effects?
16. What dose of tetanus toxin would be sufficient to kill (*a*) a mouse; (*b*) a horse, instantaneously?
17. What is meant by the period of incubation with reference to a toxin?
18. What is meant by a single fatal dose of tetanus toxin for a mouse or horse? What by a ten times fatal dose?
19. Give the exact chemical formula of tetanus toxin and antitoxin.
20. Discuss the resistance of the toxin.
21. Upon what structures of the body of susceptible beings does the toxin act quite particularly? What is tetanospasmin and tetanolysin?
22. Describe the steps in the preparation of tetanus antitoxin upon a large commercial scale.
23. What is the immunizing dose of a strong trustworthy tetanus antitoxin?
24. What is an immunizing unit of diphtheria antitoxin? What of a tetanus toxin?
25. What are the German and French standards?

CHAPTER XXIV.

BACILLI OF TYPHOID—COLON—HOG CHOLERA GROUP—BACILLUS CHOLERÆ SUIS—BACILLUS TYPHOSUS—BACILLUS COLI COMMUNIS—WHITE SCOURS IN CALVES—MALIGNANT CATARRH OF CATTLE—BACTERIUM PHLEGMASIA UBERIS—BACILLUS TYPHI MURIUM—DANYSZ'S BACILLUS PSITTACOSIS—BACTERIUM PULLORUM.

THERE is a rather large group of bacilli which have a number of common properties and the phylogenetic inter-relations of which are evidently comparatively intimate. They do not resemble each other as closely as the bacilli of the hemorrhagic septicemia group, but sufficiently to warrant their classification under one group. All are rather short, plump rods, they are generally motile, and possess a varying number of flagella; they do not form spores, they lose the stain if treated by Gram's method, they are facultative aërobics and they do not liquefy gelatin. They differ in their biologic properties as to their fermentative power toward various sugars (hexoses and disaccharids), as to their ability to form gases, acids, indol, and a number of other metabolic products, and especially as to their pathogenicity toward man and the lower animals. The following organisms, among others, belong to this group: The bacillus of hog cholera; the Bacillus coli communis, or colon bacillus; the bacillus of mouse typhoid; the bacillus of human typhoid and the paratyphoid bacillus; the various dysentery bacilli; the Bacillus enteritidis; the Bacillus fæcalis alkaligenes, etc.

THE BACILLUS OF HOG CHOLERA.

Occurrence and Historical.—The bacillus of hog cholera, Bacillus choleræ suis, or Bacillus suispestifer, was formerly believed to be the sole cause of the acute and highly contagious disease of swine, known variously as hog cholera, swine fever, pneumoenteritis, pig typhoid, cholera suum; Schweinecholera, or Schweinepest (German), and Pest du porc (French). While it has become evident that this disease in its pure and uncomplicated form is due to a filterable ultramicroscopic virus and not to a bacillus, yet the latter often infects swine during the course of hog cholera and is beyond doubt more or less pathogenic for these animals. The earliest outbreak of hog cholera was reported in Ohio in 1833, and since then the disease has spread over the entire United States. It is believed that the epidemic was

introduced from Europe, but abroad it is claimed that hog cholera originated in America and was from there transported to Europe. The hog cholera bacillus was discovered in 1880 by Salmon and Smith, and was by them proclaimed as the cause of the disease. The losses from this affection in the United States are enormous and are estimated at from ten to twenty-five million dollars annually. They are also very great in England, France, Austria, Russia, Germany, and other smaller European countries.

Pathologic Lesions.—The pathologic changes are described by Dorsett, Bolton, and McBryde as follows: "The changes seen in the internal organs vary greatly even in different animals in one and the same outbreak in a given herd. In general, these lesions may be said to be either those of a hemorrhagic septicemia or of an ulcerative enteritis, the latter particularly pronounced in the cecum and colon. The hemorrhagic lesions are characteristic of the rapidly fatal form of the disease known as acute hog cholera, the ulcerative intestinal lesions being especially prominent in those outbreaks where the animals do not succumb so rapidly; both the ulcerative and hemorrhagic lesions may, however, be seen in the same animal. When the skin of the thorax and abdomen is removed the subcutaneous areolar tissue may be found thickly dotted with ecchymoses of varying size. In acute hog cholera the inguinal glands on both sides are usually swollen and red, the hemorrhagic condition being so intense at times as to give the glands a bluish-black color. The lymphatic glands at the angles of the lower jaw may be affected in a similar manner, as may also the bronchial, mediastinal, mesenteric, mesocolic, retroperitoneal, and lumbar glands. In the chronic form of the disease the lymph glands seldom exhibit any change. The heart frequently presents at its outer surface, and also in the endocardium at times, hemorrhagic markings. The lungs, as a rule, are but slightly affected. In the acute form of hog cholera they often show ecchymoses of varying size on the serous surfaces; at times areas of bronchopneumonia or collapse are met with. In acute hog cholera the spleen, as a rule, is larger than normal, and engorged with blood, and may present numerous punctiform hemorrhages beneath the capsule, or larger hemorrhagic areas which are diffuse in character. In chronic hog cholera the spleen may be smaller than normal, and in this case the connective tissue is noticeably increased. The serous surface of the stomach may be flecked with diffuse hemorrhages, and the mucosa is not infrequently congested and inflamed. This inflammation is at times quite extensive, and may bring about destructive ulceration of the mucous membrane. Small petechiæ may be seen here and there over the mucous membrane. In acute hog cholera the chief lesions found in the intestines are ecchymoses in both serous and mucous coats, together with erosions of the mucous surfaces of both the large and the small bowels. The erosions in the cecum and colon appear to be the starting point of the button-like

ulcers which are frequently encountered in the chronic form of hog cholera. These round ulcers vary from 1 to 2 mm. to several centimeters in diameter, and are elevated above the surrounding healthy mucous membrane. They are tough and hard, and their centres are usually dark greenish-yellow in color, and in the case of the larger ulcers, all four coats of the intestine are involved. The ulcers at times are so numerous as to destroy the mucous membrane, or at least to affect it over extensive areas in the cecum and colon. The liver may exhibit extensive fatty degeneration with areas of coagulation necrosis or an increase of connective tissue. In the acute form of hog cholera, minute hemorrhages may be visible beneath the capsule. In acute hog cholera the kidneys are practically always the seat of hemorrhagic changes, which vary more or less in extent. At times the cortices are intensely congested, and all of the glomeruli

FIG. 139

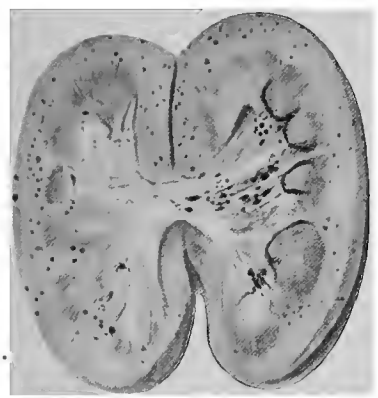
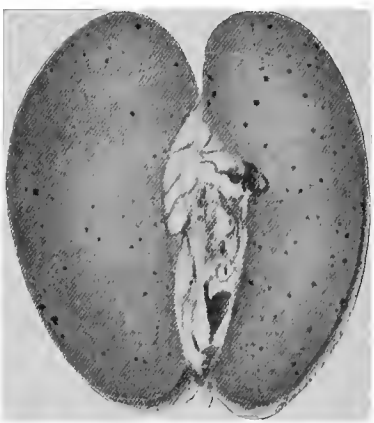


FIG. 140



Kidneys of hog in hog cholera. (Dorsett, Bolton, and McBryde.)

are visible as minute deep red points. In other instances the general congestion is absent, the major portion of the kidneys being rather paler than normal, dotted here and there with minute, sharply defined, punctate ecchymoses. At times the medullary portion of the kidneys is involved, and blood clots may be found in the pelves. In chronic hog cholera these ecchymoses are seldom seen."

In addition to the lesions which have just been described, the acute form may show nearly all of the serous membranes of the body dotted with hemorrhages. The blood and internal organs of hogs which have died of either acute or chronic hog cholera usually yield pure cultures of the Bacillus choleræ suis.

Hog cholera is conveyed from sick to healthy animals almost always, if not quite without exception, by contact, by feeding the viscera of diseased animals, and by the subcutaneous injection of the blood of

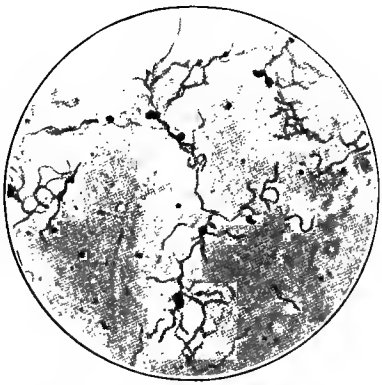
sick animals. The mortality varies from 30 to 100 per cent.; in the acute type the death rate exceeds 80 per cent. of the affected herd.

Morphology and Staining Properties.—While evidently not the cause of the disease, which can be transferred from sick to healthy hogs by the filtered, bacteria-free blood, the Bacillus suisepticus is, nevertheless, in so close a relation with the affection as it occurs under natural conditions that it must be studied in connection with it. The bacillus is a plump, rather short rod, with rounded ends; it is from 1.2 to 1.8 micra long; in the tissues it occurs as single bacilli or in chains of two, while in artificial culture media it often forms longer

FIG. 141

Bacillus of hog cholera. × 1000. (Author's preparation.)

FIG. 142



Bacillus of hog cholera, flagellar stain of Pittfield. × 1000. Cover-glass prepared by Dr. L. E. Day.

chains, which, however, rarely grow as long as those often formed by the typhoid bacillus. It is lively motile, and surrounding its body are from three to nine long flagella. It stains with the ordinary watery anilin stains, not so well with methylene blue, best with fuchsin. With the latter it appears uniformly dyed, with the former the centre often remains unstained. It is Gram negative. It does not form spores.

Cultural and Biologic Properties.—The organism grows at a wide range of temperature, namely between 8° and 42° C., best at blood temperature and in the presence or obsence of oxygen. It grows well in slightly alkaline, less vigorously in slightly acid media. It ferments glucose but not lactose or saccharose. It does not turn *milk* acid and does not coagulate it. It does not form indol. On *gelatin* plates round, bluish, transparent colonies are formed, and in gelatin stick cultures a grayish-white streak develops. The medium sometimes exhibits a milky cloudiness but is not liquefied. On *agar* slants a grayish-white glistening, not tenacious growth appears after twenty-four hours' incubation. On coagulated *blood serum* the growth is similar to that on agar. *Nutrient bouillon* becomes uniformly cloudy

and a loose sediment is soon formed at the bottom of the tube. On *potatoes* it either forms a colorless, almost invisible, moist, slightly shiny growth or one which may show a pronounced brownish tint. In *milk* the reaction after some time becomes decidedly alkaline and the fat of the medium undergoes a process of saponification, while, simultaneously, the fluid becomes opalescent, thick, and dark colored, but not viscid. It sometimes requires from three to four weeks for all these characteristic changes to become manifest. In *Dunham's peptone* water the growth is not vigorous, indol is generally not formed, but occasionally it may be formed. In the presence of glucose, the bacillus, according to Moore, during the first day forms, from the sugar in solution, from one-fourth to one-half of the total quantity of gas. By the end of the second day gas formation is nearly completed. The total amount formed is composed of carbon dioxide and hydrogen in the proportion of one volume of the former to two volumes of the latter. In the presence of glucose the reaction becomes strongly acid and the development of the organism ceases. Lactose and cane sugar (saccharose) are not fermented, and no gas is formed in their presence.

Resistance.—The organism well resists drying out, and may remain alive in dried-out tissues for several months. Alternate drying and moistening, however, kills it rapidly; also exposure to direct sunlight. It may remain alive for months in feces and moist soil; also for a long time in ordinary water. According to Preisz, it is killed at 50° C. in sixty-six hours, at 55° C. in one hour. It is killed in ten minutes or less in 1 per cent. carbolic acid, $\frac{1}{5}$ per cent. hydrochloric acid, $\frac{1}{20}$ per cent. sulphuric acid, 1 to 1000 corrosive sublimate, and 1 per cent. milk of lime; 1 to 2000 formalin solution destroys it in three hours.

Animals Susceptible.—It is pathogenic in natural infection to hogs only, but mice are quite susceptible to artificial inoculation; guinea-pigs and rabbits are less so. Very large doses injected intravenously may kill horses and cattle. Pigs, when injected subcutaneously, generally develop local lesions only, but some fatal cases after such injections have been reported.

The Etiology of Hog Cholera and the Relation of the Bacillus Choleræ Suis to this Disease.—Dorsett, Bolton, and McBryde, following up the earlier work of de Schweinitz and Dorset, came to the conclusion that pure cultures of the Bacillus choleræ suis injected subcutaneously into hogs usually produced but slight disturbance, while a severe illness frequently resulted after intravenous injections or feeding. They state that the disease produced in this manner may present the symptoms and lesions of acute hog cholera, but the contagiousness and the infectiousness of the blood are absent, and hogs which have recovered from such illness are not immune when exposed subsequently to the natural disease. They, therefore, have demonstrated that pure cultures possess a very considerable pathogenic power for

hogs, and also that the disease lacks several of the essential features of acute hog cholera.

The experiments with blood serum derived from hogs sick of hog cholera and proved to be free from Bacillus choleræ suis show, on the contrary, that such serum, upon subcutaneous injection, produces illness in hogs with great regularity, and, furthermore, that the disease thus produced possesses all the characteristics of the natural disease including symptoms, lesions, contagiousness, infectiousness of the blood, and immunity in those animals which recover. The striking contrast of these results with those obtained when cultures of the Bacillus choleræ suis are used and their complete agreement with the results obtained from unfiltered blood of sick hogs make the conclusion necessary that some virus other than Bacillus choleræ suis exists in the blood of hogs suffering from acute hog cholera, and that this virus is necessary for the production of the disease.

This virus is only known by the effects it produces. Every attempt to discover by microscopic examination or by the usual cultural methods a visible microörganism in these filtrates has failed completely. There can be no doubt that the pathogenic power of the filtered blood is due to some living agent endowed with the power of reproduction and not to the presence of a toxin alone, because the disease induced by the filtered serum is communicated from sick to healthy animals by association, and, moreover, because the disease induced by filtered serum has been transferred to a second and even a third animal by subcutaneous injections, the serum being filtered each time previous to inoculation.

While experiments by Dorsett, Bolton, and McBryde established beyond question that the filterable virus was present in all the outbreaks of hog cholera studied experimentally by these authors, it is also true that the Bacillus choleræ suis was present almost as uniformly. Even were the investigators named so inclined, it would, for this reason, be impossible to overlook the part which this organism may have played. From the results of inoculations with the filterable virus, however, and those obtained with cultures, one is compelled to conclude that the prime cause in these cases was the filterable virus, and that the Bacillus choleræ suis was at most an accessory factor.

The exact role of the Bacillus choleræ suis in outbreaks of acute hog cholera is difficult to define. That the fatal result in many instances is materially influenced by the presence of that organism cannot be doubted; in addition, the fact should be emphasized that although the filterable virus appears to have been the primary invader in the cases of acute hog cholera investigated, the possibility of independent disease being caused by Bacillus choleræ suis cannot be denied. In fact, belief in such a possiblity is difficult to avoid when the very considerable pathogenic power for hogs exhibited by many cultures of that organism when fed or administered intravenously is considered.

From the experiments of Dorsett and his co-workers it became evident that the Bacillus choleræ suis was not the true, principal, and sole cause of hog cholera. Even before the papers of de Schweinitz and Dorsett and his associates on the exact etiology of hog cholera had been published, other investigators, such as Hottinger, working in Brazil, Theiler, in South Africa, and also Boxmeyer had begun to doubt the etiological importance of the Bacillus suipestifer. Hottinger believed the bacillus to be an organism of the colon croup which penetrated from the intestines into the general circulation, but which was not the true cause of the disease. The experiments of Dorsett, McBryde, Niles, and Bolton, of the United States Department of Agriculture on the production of the disease by bacteria-free blood, were confirmed by Theiler, Hutyra, Ostertag and Stadies, McClintock, Boxmeyer and Siffer, Uhlenhut, Xylander and Bohtz, Wassermann, Carre, Leclainche and Vallee, and others. There is at the present time no doubt remaining that hog cholera is, indeed, due to a filterable, invisible, ultramicroscopic, highly contagious living virus, and not to the bacillus suipestifer.

Protective Inoculation.—The fact that animals which recover from hog cholera are immune for the remainder of their natural lives was noticed early in the modern studies of the disease. Such animals could not even be made sick by injecting into them blood from cholera-sick hogs. Since the discovery that the affection is due to an invisible virus, Dorsett has worked out a method to hyperimmunize hogs, so that their blood contains a large amount of antibodies and can be used as an antitoxin or immune serum to protect non-immune animals against a fatal or serious attack. The serum is prepared in a hog which has survived a natural attack of the disease. The animal is injected with very virulent blood from a cholera-sick hog, but it must first be definitely ascertained by the injection of 2 to 5 c.c. of the blood into two non-immune hogs, that it actually is virulent. If the injected non-immunes do not become very sick themselves, the blood tested is not of sufficient virulence, and the test must be repeated until found satisfactory with virulent blood from another source. The hyperimmunization of the immune hog is then brought about in the following manner:

1. *Subcutaneous Injections.*—(*a*) Inject the immune subcutaneously with defibrinated disease-producing blood in the proportion of 10 c.c. for each pound of body weight; or (*b*) inject the immune subcutaneously with 1 c.c. of defibrinated disease-producing blood for each pound of body weight. After an interval of one week give a second injection of 2.5 c.c. disease-producing blood for each pound of body weight. After the interval of another week give a third injection of 5 c.c. of disease-producing blood for each pound of body weight.

2. *Intravenous Injections.*—(*a*) Inject the immune intravenously with defibrinated disease-producing blood in the proportion of 5 c.c. of blood for each pound of body weight; or (*b*) inject the immune

intravenously with defibrinated disease-producing blood in the proportion of 5 c.c. of blood for each pound of body weight, and after an interval of a week, if the hog has recovered, repeat the injection.

3. *Intra-abdominal Injections.*—Inject the immune intra-abdominally with defibrinated disease-producing blood in the proportion of 10 c.c. of blood for each pound of body weight.

The danger of spreading the disease by immune-infected hogs disappears within twenty-four hours after an injection, because after this lapse of time, as has been shown by Uhlenhut, their drawn blood cannot transfer the disease; evidently the virus has been neutralized in the body of the immune animal. After an immune hog has recovered from the effects of the last injection of the virulent blood (generally in eight to ten days) its blood can be drawn to inject and immunize unprotected hogs. Blood may be drawn from the hyperimmunized animal by severing the carotid artery and bleeding it to death or by cutting off the tail. The latter method is preferable for the first drawings, as the bleeding may be stopped at any time, thus permitting the immune animal to live and to be used again to procure further supplies of blood. Experiments have shown that after hyperimmunization blood may be drawn from the tail three or four times at intervals of a week, without perceptibly lessening the antitoxic properties of the serum obtained. One week after the last collection of blood the serum animal may be killed by severing the carotid and collecting all the blood that can be obtained. The serum of the blood is separated from the clot in the usual manner after coagulation has taken place. A fraction of a per cent. of carbolic acid is added to the serum. Different sera obtained are generally mixed and the efficacy of the mixture is then tested as follows: Eight young pigs weighing from thirty to sixty pounds receive subcutaneously each 2 c.c. of blood from an acute case of hog cholera, two of the pigs treated remain unprotected and the other six are protected in groups of two by the injection, respectively, of 10 c.c., 15 c.c., and 20 c.c. of the immune serum. A good immune serum should protect in a dose of 15 c.c. or less. If this is found to be the case, 20 c.c. of the serum is used as an immunizing dose to protect young pigs weighing from twenty to one hundred pounds. The injection is generally made on the inside of the hind leg. The passive immunity so produced lasts for several weeks only; to produce a more lasting effect, however, the simultaneous method is used in which 20 c.c. of the immune serum is injected on one side of the animal, while the other receives a small amount (about 2 c.c.) of virulent blood from a case of hog cholera.

Agglutination of Bacillus Suipestifer by the Serum of Hogs Hyperimmunized against Hog Cholera.—The first tests to ascertain whether the serum of hogs sick with hog cholera would agglutinate the hog cholera bacillus in high dilutions were made by Dinwiddie. His results were negative. McClintock, Boxmeyer, and Siffer found that the serum of normal hogs agglutinated the hog cholera bacillus in a

dilution of 1 to 250 and that the agglutinative power was much incrased after inoculation with hog-cholera vaccine; it could be shown that the intraperitoneal injection of such vaccines was usually followed by the production of large quantities of agglutinins. The amount of the vaccine, however, had no relation to the amount of agglutinins formed. Giltner has recently published a preliminary report on a series of experiments which show that the serum of hogs hyperimmunized against hog cholera by the Dorsett-Niles method has a very high agglutinative value for the hog-cholera bacillus. He found values as high as 1 in 2000. The blood serum of immune hogs (which had not yet been hyperimmunized) agglutinated in dilutions as high as 1 in 1000. These results are really not very astonishing when the fact is considered that hog-cholera bacilli are, as a rule, found in the blood and organs of animals sick from hog cholera. For this reason, an animal which survives an attack of the disease may be expected to possess a certain amount of immunity against the hog-cholera bacillus as well as against the invisible filterable virus. The presence of a large amount of agglutinins against the cholera bacillus in hyperimmunized animals can likewise be easily accounted for by the fact that with the large amount of virulent blood injected for hyperimmunization a considerable number of hog-cholera bacilli are also injected. The agglutination tests with the hog-cholera bacillus are made as macroscopic tests, and the method is identical with that employed in the case of the glanders bacillus. According to a circular of the United States Department of Agriculture the *Bruschettini Hog-cholera Vaccine* and the *Bruschettini Hog-cholera and Swine-plague Serum* are without value whatsoever. The department reported that healthy pigs were injected with the serum and were exposed after twenty-four hours by being placed in the same pens with hogs affected with the disease. All the hogs treated with the Bruschettini serum contracted hog cholera within the usual period of time after exposure and finally died, exhibiting typical lesions of hog cholera at autopsy.

BACILLUS TYPHOSUS.

Occurrence.—The best known of the microörganisms of the group under discussion is the typhoid bacillus. It is the cause of typhoid fever, also called abdominal typhoid or enteric fever in man. While pathologic changes and death can be produced in experimental animals by the inoculation of typhoid bacilli, none of the lower animals are susceptible to a natural typhoid infection. In man, typhoid fever is, as a rule, contracted through the drinking water or food which has directly or indirectly become contaminated with the excrements of patients suffering from the disease. The bacillus, after ingestion with water or food, multiplies enormously on and in the

mucosa of the small intestine, where it causes local pathologic changes, such as swelling and ulceration of the solitary and agminated lymph follicles, and general symptoms due to the absorption of its toxins. At a later stage it enters the general circulation, becomes localized in the spleen and also in other places, preferably in the lungs, and is then excreted not merely with the feces, but also with the urine, sputum, etc. It is difficult to isolate the bacillus from the feces, less difficult from the urine, and easiest from the juice of the spleen, provided it is obtained in a perfectly aseptic manner. As a rule, typhoid bacilli disappear from the urine and feces within a few weeks after the termination of the disease; they generally, however, remain present for a long time in the gall-bladder. In exceptional cases persons who have had typhoid fever may void the bacilli with their feces for years, becoming in this way a constant source of spreading typhoid through contaminating milk or water. One attack of typhoid fever, as a rule, induces immunity for the remainder of the individual's life, but in about 2 per cent. of the cases a second attack, generally of a mild character, has occurred. Oysters obtained from water contaminated by sewage may harbor virulent typhoid bacilli, and when eaten raw, cause infection.

The Typhoid Culture Medium of Drigalski and Conradi.—From a hygienic standpoint it is sometimes of great importance to be able to decide whether drinking water, milk, oysters, etc., or the stool of a supposed permanent typhoid carrier contain typhoid bacilli, and in order to find them rapidly a special medium is required. This is prepared as follows: Three pounds of chopped lean beef are allowed to stand with two liters of water in the cold (ice-box in summer) for twenty-four hours. The meat infusion is then boiled for one hour and filtered. Add 20 grams of Witte's dry peptone, 30 grams of nutrose, and 10 grams of common salt; boil another hour; filter again. Now add 60 grams of agar, boil several hours, neutralize with caustic soda solution, and filter clear in the steam sterilizer or hot-water funnel. Take 300 c.c. Kahlbaum's litmus solution, add 30 grams lactose, and boil for fifteen minutes. Mix the fluid agar with the litmus-lactose solution (the mixture will generally turn red); it is now faintly alkalinized with a 10 per cent. soda solution. Finally, add 4 c.c. hot sterile 10 per cent. soda solution and 30 c.c. of a sterile solution (1 to 1000) of crystal violet (Hoeschst B). This medium is distributed into sterile test-tubes. When the search for typhoid bacilli is made, plates are poured into Petri dishes from the melted medium, and after the latter has set, the surface is inoculated from the suspected material. If the latter is a stool, it must be diluted with nine times its volume of 0.85 salt solution. The plates are then inverted and allowed to stand slightly open for one-half hour to permit the surface to dry somewhat. They are then placed in an inverted position in the incubator and examined after sixteen to twenty-four hours, when the typhoid bacilli, if such have been present, appear as small, transparent

blue colonies, about 1 to 3 millimeters in diameter. Colonies of the colon bacillus and other members of the colon-typhoid group are larger, coarser, less transparent, and red. To identify the bacilli beyond doubt the small transparent colonies must be tested with an agglutinating typhoid serum.

Morphology.—The typhoid bacillus is a rather plump rod 1 to 3 micra in length, 0.5 to 0.8 micron in diameter. It has rounded ends, frequently forms longer or shorter chains, is very actively motile, and possesses numerous long, delicate flagella, but does not form spores. It stains with the ordinary watery anilin stains, but takes them rather slowly, and is best dyed with a watery fuchsin solution; it is Gram negative.

Cultural Properties.—The bacillus grows both in the presence and absence of oxygen, best at blood temperature, well between 25° and 37° C., poorly below 20° C. It is easily destroyed by antiseptics and by heat. It grows well on all of the ordinary laboratory media and does not liquefy gelatin. On *potatoes* it generally forms an abundant but invisible growth; occasionally, however, a heavy, yellowish-brown, visible growth. The typhoid bacillus does not form gas from glucose, galactose, or levulose; it does not coagulate milk.

Formation of Agglutinins and Other Antibodies.—When typhoid bacilli are inoculated into animals, or when human beings become infected in a natural way, through drinking contaminated water or otherwise, antibodies which will precipitate, agglutinate, and dissolve typhoid bacilli develop in the blood serum during the course of the disease. In other words, the blood serum then contains specific precipitins, agglutinins, and bacteriolysins. These antibodies appear comparatively early, and for this reason an early and indubitable diagnosis of typhoid fever in man can today, as a rule, be established by the serum test. This very simple test is usually made as a microscopic test, and the only elements necessary are some blood from the suspected case and a young, vigorous, typhoid-bacillus culture. The latter is generally raised on slanted agar or glycerin agar in the incubator at blood temperature (37° C). It is best to use a culture eighteen hours old for the test; at least it should not be much older than twenty-four hours, because young cultures contain the most vigorous and the most lively bacilli. Blood is obtained by puncturing the cleansed ear or finger tip of the patient with a sharp sterile needle and allowing it to drop (in small drops) on a clean slide, so that the individual drops do not touch but have a certain free space between them. The blood is then allowed to dry on the glass slide, which, if it is not to be used immediately, should be wrapped in a clean piece of paper and protected against moisture and dirt. The test itself can be made at any time from this dried blood. The steps are as follows:

1. Remove from the agar slant with a platinum loop some of the growth, preferably from the very margin, because the most motile

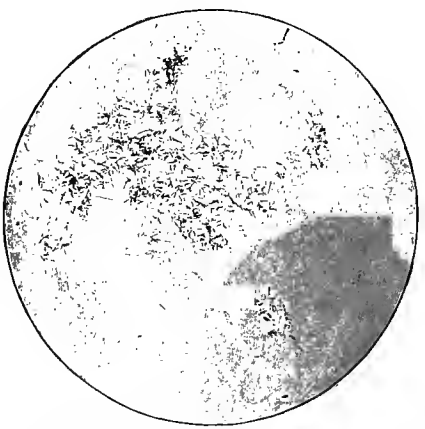
typhoid bacilli are found here. Rub up the loopful of bacteria in a clean watch-glass with a sufficient quantity of distilled water to make an even milky but not very concentrated emulsion. Place a small drop of this emulsion on a clean cover-glass, add another drop of water with the platinum loop, and again mix thoroughly until an even, homogeneous emulsion is obtained. Place the cover-glass over a concave slide, around the concavity of which vaselin has been painted. Examine this hanging drop with oil-immersion magnification to ascertain that the typhoid bacilli are lively, motile, and not clumped.

2. Prepare another cover-glass by placing on it a small drop of the typhoid bacilli emulsion in the watch-glass.

FIG. 143

Typhoid bacilli from nutrient gelatin. × 1100. (Park.)

FIG. 144



Gruber-Widal reaction. Bacilli gathered into one large and two small clumps, the few isolated bacteria being motionless or almost so.

3. Mix a small drop of dried blood on the cover-glass with about 10 to 20 drops of distilled water, which are added with the platinum loop. Then rub up well with the dried blood, which, of course, is now dissolved out by the water. This mixing is done directly on the slide on which the blood was collected and allowed to dry.

4. Add to the emulsion on the second cover-glass, with platinum loop, a small drop of the dilute blood, mix well, and place cover-glass on a concave slide.

There are now two hanging-drop preparations: the first one is simply an emulsion of young typhoid bacilli in distilled water, which serves as a control for the second, which contains bacilli and distilled water together with diluted blood from the suscepted cases of typhoid fever. If this case is indeed one of typhoid fever it will be noted after fifteen to thirty minutes (sometimes even earlier) that the typhoid bacilli mixed with the dilute blood lose their motility, become glued to each other in little masses, and finally become indistinct, while

some of them are actually dissolved. This process is spoken of as the immobilization, agglutination, and solution of the bacilli. This serum test is known as the *Gruber-Widal test.* The identical principle is employed in a microscopic or a macroscopic test in glanders, although in this case much higher dilutions must be used (see chapter on Glanders). The test and control test are frequently made on a single slide with two concavities. The hanging drop with water only is placed over one and the hanging drop containing the dilute blood serum over the other. In making the test in the manner described above, care must always be taken to have young motile typhoid bacilli and to dilute the blood sufficiently. If too strong a concentration of the human serum is used, a certain amount of immobilization and agglutination will occur, and this may simulate a positive reaction. The student must also remember that little masses of blood corpuscles or fibrin are found in dried and redissolved blood; these must not be mistaken for agglutinated bacilli. In a dilution of 1 in 20 of the dried blood the reaction should begin to manifest itself in about twenty minutes; in a dilution of 1 in 10 in less than fifteen minutes, often in five minutes. When the reaction with the 1 in 10 dilution is doubtful lesser dilutions should always be made. A positive test is not found during the very first days of the disease, but about 20 per cent. of cases of typhoid give a positive reaction during the first week and 90 per cent. in the fourth week. Some cases of typhoid never give a positive reaction either during or after the disease. The author has seen one case in which a positive reaction was only obtained on a single day during the course of the disease (third or fourth week), never before, never afterward. Sometimes a positive reaction persists for years after the disease has run its course and some permanent typhoid carriers may give a positive reaction as long as they harbor bacilli.

The Widal-Gruber test for typhoid fever has been used in hundreds of thousands of cases throughout the world. If made with the necessary precautions it is a most excellent and trustworthy test, but it is not absolutely infallible on each and every application.

The agglutination test in typhoid fever has been so fully explained in all its details because it is the best known and most extensively used microscopic agglutination test. Its principle and technique is applicable to many microbic infections in animals, perhaps less in practice but extensively in scientific laboratory work in the investigation of problems of immunization in animal diseases.

BACILLUS COLI COMMUNIS.

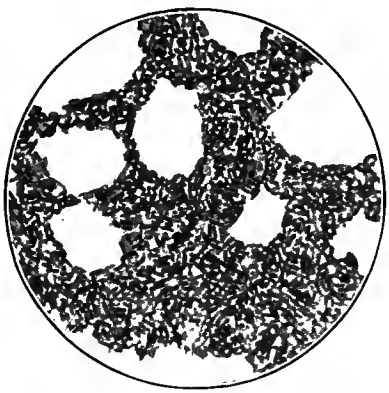
Occurrence.—The Bacillus coli communis, or Bacillus coli or colon bacillus, was first found in Naples by Emmerich in the organs and blood of patients who had died of Asiatic cholera. Emmerich thought

that he had discovered the cause of this disease, and named the microorganism Bacillus Neapolitanus. In the following year Escherich demonstrated the bacillus in the stools of normal milk-fed infants, and it has since been found as an entirely normal inhabitant in the intestines of man and most of the domestic and wild animals. It is, however, claimed that the bacillus does not occur in the intestines of the horse. The organism is found widespread in nature as a saprophyte. Some authors, like Flügge, strongly maintain that the organism is ubiquitous and occurs extensively in air, soil, water, and independent of contamination from human feces or animal manure. Escherich and Pfaundler, conceding its widespread prevalence in the outside world, incline to the belief that it is generally, directly or indirectly, derived from fecal matter. They admit, however, that the bacillus has also been found in moderate numbers in water, which, according to chemical analysis, was absolutely pure and unobjectionable and showed no chemical evidence of contamination with sewage. Weisenfeld, in 56 specimens of water, both good and bad, always found the Bacillus coli, and claims, for this reason, that the presence of this organism cannot be used as an index of contamination with sewage. The same standpoint was previously taken by Kruse, Beckmann, Pajal, Miquel, Schumann, and, as stated, by Flügge. The question has been here discussed because occasionally it becomes a matter of dispute whether milk and other foods containing the colon bacillus must be considered as contaminated with fecal matter or not. There is no doubt that enormous numbers of the colon bacillus occur in the intestines of cattle, as it is found in great numbers in the intestines of man. In both man and animals the bacillus is ordinarily not pathogenic, but in fact beneficial. It cannot split up and produce putrefactive changes in native albumins, but it splits up carbohydrates (starches and sugars). It is quite evident that it plays a physiologic and beneficial part in the intestines and prevents excessive putrefactive processes. While actual counts of the colon bacillus in the feces of animals have not been made, it has been ascertained by Eberle and Lange that 1500 to 3500 millions per gram of feces are present in the stool of milk-fed healthy infants, and according to Sucksdorf's estimation the figure in adults is still 381,000,000 per gram. Ordinarily the Bacillus coli, as stated, is not pathogenic, but it may, in consequence of inflammatory processes, leave the lumen of the intestines and wander through the damaged wall of the gut into the peritoneal cavity, the bladder, the ovary, the kidneys, etc., producing either alone or in combination with other bacteria serious pathologic conditions.

Morphology and Staining Properties.—The Bacillus coli is generally a short, rather plump rod, 2 to 4 micra long (sometimes as long as 6 micra), and from 0.4 to 0.7 micron wide, with rounded ends. In culture media and also in tissues which it has invaded in a pathogenic manner it sometimes becomes very short, so that it resembles a coccus. It occurs singly or in pairs, and in culture media also in longer

chains. It does not form spores, but sometimes has a capsule; it is rather sluggishly motile, and possesses flagella, generally four to eight, sometimes as few as two, but, according to Loeffler, often as many as ten to twenty. The flagella are generally shorter and thinner than those of the hog cholera bacillus. The bacillus stains with the ordinary watery anilin stains, best with fuchsin; it sometimes shows deeper stained polar granules and unstained portions in the centre. It is Gram negative, like all the other bacilli of the hog-cholera-typhoid-colon group.

FIG. 145



Bacillus coli communis. × 1000.
(Author's preparation.)

FIG. 146

Shiga's dysentery bacillus. × 1000.
(Author's preparation.)

Cultural Properties.—It grows best aërobically, but also anaërobically at temperatures from 10° to 37° C., and particularly well at blood temperature. On *gelatin* plates the colonies appear after eighteen to twenty-four hours. They are grayish white, opaque, and moist; in the depth of the medium they are finely granular and yellowish; later they become larger, more coarsely granular, and darker. They are round, oval, or whet-stone like. The gelatin is not liquefied. In stick cultures the development is nail-like, and the surface growth becomes relatively abundant, covering the whole surface. On *agar* the organism forms a grayish-white, rather moist growth. It clouds *nutrient bouillon* rapidly, and sometimes forms a pellicle. On *potatoes* an abundant development rapidly occurs; it is yellowish and moist, and markedly elevated. The growth on coagulated *blood serum* is much like that on agar. *Milk* is coagulated in consequence of the formation of lactic, acetic, formic, and succinic acids. The colon bacillus ferments glucose, lactose, maltose, saccharose as well as other carbohydrates, and in their presence forms carbon dioxide and hydrogen, generally in the proportion of one to two. Some varieties of the colon bacillus vary considerably as to the amount and proportion

of gas formation. In *Dunham's peptone* solution the bacillus forms indol. The term Bacillus coli does not cover, as it is becoming more and more evident, a single species, but a number of closely allied varieties.

WHITE SCOURS, OR DIARRHEA, IN CALVES.

Occurrence and Historical.—White scours, or diarrhea, in calves, dysenteria neonatorum, diarrhœa neonatorum; "Ruhr der Säuglinge," "Durchfall des Säuglinge," "Kälberruhr" (German), is an acute, contagious diarrhea of calves, attacking them during the first days of their lives. The disease has occasionally also been observed among foals, lambs, and pigs. The disease in calves is generally caused by bacteria representing different varieties of the colon bacillus. This was established through the long-continued investigations and experiments of Jensen, who was the first to make a bacteriologic study of the disease (1891). His early findings of pathogenic colon bacilli in the blood and organs of calves dead from the disease was first confirmed by Pina, Monti, and Veratti.

The disease generally begins one to two days after birth, sometimes within a few hours after delivery. After two to four further days the clinical picture of the disease is well developed.

Pathologic Lesions.—Two forms of the disease, varying in rapidity of the course and the pathologic lesions, can be distinguished. In the rapidly fatal form the mucosa of the stomach and intestines is red and hyperemic, hemorrhages are seen here and there, and the contents of the intestines are hemorrhagic. The peritoneal covering of the intestines is likewise hyperemic. The mesenteric glands are swollen, hyperemic, and edematous. The spleen is generally enlarged, sometimes considerably. In the blood, colon bacilli are found in considerable numbers. In the second type, which generally appears somewhat later after birth, and which takes a somewhat slower course, the hyperemia of the gastro-intestinal tract and of the internal organs in general is not so great, but of a rather moderate degree. The intestines are flabby, pale, extended by gas; the intestinal mucosa is only very moderately hyperemic. The mesenteric glands are swollen, but pale; the spleen is not swollen. Colon bacilli are not found in the blood, but in the intestines only.

Diarrhea in calves has also been sometimes ascribed to bacilli of the colon group, which are more nearly related to the hog cholera than to the colon bacillus. Jensen also observed some cases of diarrhea in calves which he considered due to an infection with the Bacillus proteus vulgaris. All these cases, except those apparently due to pathogenic varieties of the colon bacillus, however, are rare; the latter have also been designated as *"coli bacillosis of calves."* Moore, likewise, has found a variety of the colon bacillus as the

cause of diarrhea in calves, while Nocard, who studied an epidemic of white scours among calves in Ireland, attributed it to a bacillus of the hemorrhagic septicemia group.

MALIGNANT CATARRH OF CATTLE.

This disease, also known as rhinitis gangrænosa, "Bösartige Kopfkrankheit (German), mal de tête de contagion (French), is an acute, non-contagious disease of cattle and buffaloes, involving particularly the mucous membranes of the head in which pseudomembranes and ulcerations accompanied by profound nervous disturbances are produced. The disease has been described in most countries of Europe and in South Africa in cattle, and in India and Java in water buffaloes or carabaos. Leclainche, in 1898, reported a bacterium with the characters of the colon bacillus as the cause of the disease; in experimental work the organism proved to be pathogenic for cattle, rabbits, and guinea-pigs. The French investigator found these virulent bacilli in the intestines and mesenteric glands, and sometimes also on the nasal mucous membrane, the papillæ of the tongue, and the sublingual glands. The bacillus isolated by Leclainche evidently forms a very toxic substance in bouillon cultures, and if 2 c.c. of such a growth is injected into young cattle, profound but transitory nervous symptoms, such as restlessness, trembling, elevation of temperature, colicky pains, running from the nose, and watering of the eyes are produced. Leclainche's investigations have not yet been confirmed by others.

BACTERIUM PHLEGMASIA UBERIS.

This name was given by Kitt to a bacillus which he isolated in 1886 from cases of inflammation of the udder in cows (parenchymatous mastitis). It is now known that the bacterium of Kitt is identical with the colon bacillus.

BACILLUS TYPHI MURIUM.

This organism, also known as the bacillus of mouse typhoid, first isolated from the blood of mice by Loeffler, is a member of the colon group. It grows in the presence or absence of oxygen, stains well with the watery anilin stains, but not by Gram's method; it does not liquefy gelatin, and forms acid in milk without coagulating the medium. The organism is not pathogenic to the ordinary domestic animals, either mammals or birds. It kills mice, however, in from eight to ten days when introduced into their intestinal canal; when injected subcutaneously it kills them in one or two days. The bacillus has been used a number of times to rid large areas of mice; the most successful attempt of this kind was made by Loeffler in Greece, where he succeeded in ridding the country of a pest of field mice.

BACILLUS OF DANYSZ.

A bacillus either very similar to or identical with the Bacillus typhi murium was discovered by Danysz, and is known by the name of the discoverer. It has been much exploited commercially as a destroyer of rats. According to Rosenau's experiments the so-called Danysz-virus and similar preparations are worthless in the destruction of rats.

PSITTACOSIS OR SEPTICEMIA OF PARROTS.

This disease of African and American species of parrots, also called mycosis of parrots, is of interest because it is claimed that it is communicable to man, producing in persons exposed to the contagion a frequently fatal type of pneumonia. The disease in parrots is characterized by prostration and diarrhea, and in its course small grayish-white nodules are formed in the liver and other internal organs. The bacteriology of the disease has been studied by Nocard, who describes a bacterium belonging to the colon group as its cause. It grows both aërobically and anaërobically on the ordinary culture media. In *bouillon* it grows rapidly in the incubator, clouds the medium uniformly, and forms a thin surface pellicle which sinks to the bottom of the tube on slight agitation. On *gelatin* it forms light bluish, shining, and also darker opaque porcelain white colonies. It grows also on *agar*, on *potatoes*, in *milk*. Gelatin is not liquefied, lactose not fermented, and milk not coagulated by it. The bacillus of psittacosis, therefore, in cultures acts more like the typhoid or hog-cholera bacillus than the Bacillus coli. According to Dupuy's statistics, as quoted by Nocard, seventy persons living in Paris during the years 1892 to 1897 contracted psittacosis from parrots, and twenty-four of these died. It is believed that the disease is generally contracted by persons kissing the sick parrots or by otherwise coming in too close contact with them. The first cases of psittacosis pneumonia in man were probably observed by Ritter in Switzerland in 1879. Palamidessi observed five cases in one family in Florence. Leichtenstern, who saw some cases of this peculiar pneumonia in man in Germany, doubts that they have their origin in parrots suffering from psittacosis.

BACTERIUM PULLORUM.

Occurrence.—An epidemic disease with a very high mortality occurs among very young chicks recently hatched. The young fowls first show a loss of appetite and sluggishness, the feathers become ruffled, and diarrhea appears; the droppings are of a whitish color. The disease is known under the name of *white diarrhea of chicks*. As

the cause of this affection, Rettger and Harvey have described a specific organism, which, according to its morphologic and cultural properties, belongs to the colon-hog-cholera group of bacteria.

Pathologic Lesions.—The chicks on postmortem examination are found to be much emaciated; the crop is empty, the intestines are pale, but without indications of ulceration or congestion, and the liver is pale, with the exception of a few patches and streaks which are of a dark color, while the spleen, lungs, and kidneys appear normal. In stained sections of the tissues small slender bacilli are occasionally found. These organisms do not occur in groups, but singly, scattered here and there through the section. While in healthy chicks or in those which have died from other causes the yolk is generally completely absorbed at this age, in chicks dead from white diarrhea the yolk sacs are not yet absorbed, but are present, varying in size from a small pea to an Italian chestnut. According to Rettger and Harvey the best method of obtaining the organism in first culture is to open the body with a sterile knife, then remove as much blood as possible with a sterile platinum loop; also pieces of spleen or liver, or some of the contents of the unabsorbed yolk sac, and make streaks on agar slants. The latter are to be incubated at 35° to 37° C. In the course of twenty-four hours, inspection, preferably with a hand magnifying lens, will reveal the presence of minute colonies which resemble small droplets of fat. The colonies are discrete, and remain so even after several days of incubation. The colonies never grow to be large, although there is considerable increase in size after the first twenty-four hours. The growth on *agar* streaked with the infected blood during the first twenty-four to thirty-six hours has all the appearances of the ordinary streptococcus.

Morphology.—The Bacterium pullorum, isolated by Rettger and Harvey from white diarrhea, or septicemia of chicks, is a long, slender bacillus, 3 to 5 micra long by 1 to 1.5 micra wide, with slightly rounded ends. It usually occurs singly, chains of more than two bacilli being rarely found. It is non-motile, and resembles the bacillus of typhoid fever. It is stained readily by the ordinary watery anilin stains, and is Gram negative. It does not form spores.

Cultural Properties.—The colonies on agar slants have been already described. On *gelatin* plates it forms surface colonies which somewhat resemble the grape-leaf colonies of the typhoid bacillus. In gelatin stick cultures a delicate growth appears in forty-eight hours along the whole line of inoculation; the growth is distinctly granular in appearance, and spreads very little on the surface. Gelatin is not liquefied. The development on *potato* is slow; in *litmus milk* there is no change for forty-eight hours, then the medium becomes slightly acidified, but there is no coagulation. The organism can split dextrose and mannite with acid and gas production, but it does not ferment either maltose, lactose, saccharose, inulin, or dextrin. It does not form indol or nitrite in *Dunham's solution.*

Rettger and Harvey inoculated a number of chicks with three stems of their bacillus obtained from three different epidemics. Most inoculations were successful and led to a typical fatal attack of white diarrhea. From the dead chicks the organism could again be obtained in pure cultures. A few feeding experiments also gave positive results.

Morse, who studied white diarrhea in chicks before the publication of Rettger and Harvey's work, came to the conclusion that the affection was due to a protozoan organism, *i. e.*, Coccidium tenellum.

QUESTIONS.

1. What bacteria belong to the typhoid-colon-hog cholera group of bacilli?
2. Name some of their common properties.
3. In what respect do they differ from each other?
4. What other names have been given to the disease hog cholera?
5. What is the hog-cholera bacillus or Bacillus suipestifer?
6. Discuss the geographical occurrence of hog cholera.
7. Describe in general terms the pathologic lesions of the disease.
8. Describe the lymphatic glands in the disease; also the changes found in the gastro-intestinal tract.
9. Describe the appearance of the kidneys.
10. Describe the morphology and the staining properties of the Bacillus choleræ suis.
11. At what temperature does this organism grow?
12. What sugars are fermented by this bacillus?
13. Describe its growth on gelatin plates and in gelatin stick culture.
14. How does it act when growing in milk? How in bouillon?
15. Does it form indol?
16. Give the differential features of Bacillus suipestifer and Bacillus (bipolaris) suisepticus.
17. Discuss the resistance of Bacillus choleræ suis.
18. Give a definition of the term filterable, invisible, ultramicroscopic virus.
19. Describe the experiences and experiments which have led to the recognition of the fact that hog cholera is not due to the Bacillus choleræ suis.
20. Describe the method of hyperimmunizing hogs against hog cholera to obtain an immune or antiserum of high value.
21. What is an immune hog?
22. How is hog-cholera blood tested as to its virulency?
23. How soon is the virulent blood neutralized in the body of an immune hog?
24. Describe methods of obtaining the immune serum from the hyperimmunized hog.
25. Describe the method of testing the immunizing or protecting power of the immune serum.
26. Describe the method of procuring passive immunity in non-immunes.
27. Describe the simultaneous method of producing a more lasting active immunity.
28. In what dilution does the blood serum from a healthy hog agglutinate the hog-cholera bacillus?
29. What effect has the intraperitoneal injection of vaccines prepared from the Bacillus choleræ suis upon the agglutinating power of hog's blood toward this organism?
30. How does the blood serum of hogs hyperimmunized against hog cholera behave toward the Bacillus choleræ suis?
31. What human and what animal diseases are caused by the Bacillus typhosus?
32. How is typhoid fever spread?
33. How can typhoid bacilli be detected in milk or water?
34. Describe the preparation of the Drigalski-Conradi medium.
35. How is it used? How do typhoid cultures look on it?
36. Describe the morphology of the typhoid bacillus.

37. Describe its cultural properties.
38. How does it generally grow on potatoes?
39. How does it act toward milk; toward different sugars in solution in the culture media?
40. What kind of immune bodies (antibodies) are formed in typhoid infection in man and animals?
41. What is the Gruber-Widal serum test for typhoid fever?
42. What apparatus, cultures, reagents are necessary to make the test?
43. What kind of a culture is to be used; how is the blood to be obtained and treated?
44. Describe in detail the steps to make the test. What is the outcome in a positive, and what in a negative test?
45. Discuss the advantages and the shortcomings of the test.
46. What is meant by the term permanent typhoid bacilli carrier? (The German word for this is "Dauerausscheider.")
47. Why should the veterinary student become familiar with the technique of the Gruber-Widal test in typhoid?
48. Give the other names in use for the colon bacillus.
49. Where is this bacillus found in connection with animal life? Where is it found in the outside world?
50. Is its presence always indicative of contamination with feces, manure, or sewage? What are the views held by different authors on this point?
51. Under what conditions may the colon bacillus become pathogenic?
52. Describe the morphology of the bacillus.
53. Describe its growth in gelatin, in milk, in bouillon, and on potatoes.
54. What effect has it on various sugars, such as maltose, glucose, lactose?
55. What are its characteristics as to gas and indol production?
56. Give the differences in the cultural properties of the colon, the hog cholera, and the typhoid bacillus.
57. What are the other names under which white scours in calves is known?
58. What is probably the cause of this disease?
59. Describe the pathologic lesions in (*a*) a very rapid case; (*b*) a less rapid case.
60. What other bacteria may be the cause of diarrhea in calves?
61. What is malignant catarrh in cattle? What is the organism that Leclainche claims is its cause?
62. What is psittacosis?
63. What microörganism causes it?
64. Is man susceptible to this disease?
65. Describe the Bacillus typhi murium. What is Danysz's bacillus?
66. What are the principal symptoms and the chief pathologic lesions of white diarrhea in chicks?
67. What, according to Rettger and Harvey, is the cause of the disease?
68. Describe the morphology of the Bacterium pullorum.
69. How can it best be obtained from animals dead from the disease?
70. Describe its cultural properties.
71. What effect has the Bacterium pullorum when fed to or inoculated into very young chicks?
72. What is the relation of Coccidium tenellum to white diarrhea of chicks?

CHAPTER XXV.

BACILLUS OF SWINE ERYSIPELAS—BACILLUS OF MOUSE SEPTICEMIA.

BACILLUS OF SWINE ERYSIPELAS.

Occurrence and Historical.—The disease swine erysipelas, red fever of swine; "Backsteinblattern," "Stäbchenrothlauf" (German); "rouget du porc" (French), is an acute septicemic disease of swine occurring either sporadically or epizoötically. The disease is comparatively prevalent in European countries. In Germany, 89,087 cases, with a mortality of over 80 per cent., were reported in 1903; in France the annual loss is estimated at 100,000 animals and over, and the disease is also relatively prevalent in other countries of Europe. The affection has been known for a long time, but prior to the investigations of Pasteur and Thuillier, who, however, did not discover its true cause, it was mistaken for anthrax in swine. The true cause of the disease was found in 1885 by Loeffler and a little later also by Schütz in a bacillus known as the *Bacillus erysipelatis suis* or the *bacillus of swine erysipelas.*

Pathologic Lesions.—In the *acute form* of the disease the post-mortem findings are not very characteristic. The mucosa of the stomach, particularly in the neighborhood of the pylorus, is inflamed, swollen, and red, covered with a tenacious mucus, upon the removal of which numerous hemorrhagic spots are encountered. The mucosa of the small intestines is likewise inflamed and congested, Peyer's patches are swollen, and here and there superficial ulceration is seen. The latter also occurs in the large intestines, particularly in the region of the ileocecal valve. The spleen is congested and often somewhat enlarged, and the kidneys show cloudy swelling. In the cortical substance of the latter, reddish points are seen. These are the inflamed and hemorrhagic glomeruli (glomerulonephritis). The lungs are hyperemic and edematous. The lymphatic glands are edematous, hyperemic, and much swollen. The serous membranes sometimes show a fine fibrinous deposit, and frequently hemorrhagic spots. The changes in the skin from which the disease has received its name consist in hemorrhagic spots, which are due to the great congestion of superficial bloodvessels with some blood extravasation into the subcutaneous connective tissue.

In the *chronic form* of the disease an inflammation of the serous

lining of the interior of the heart is frequently found. The valves are covered with fibrinous and hemorrhagic wart-like deposits, and scattered ulcerations are seen. This is a pathologic change known as *endocarditis verrucosa* and *ulcerosa*. The specific bacillus causing the disease occurs in moderate numbers in the blood and in larger numbers in the spleen, liver, and kidneys, and the verrucous deposits in the heart.

Morphology and Staining Properties.—The Bacillus rhusiopathiæ suis, or bacillus of swine erysipelas, can be best seen in preparations made from the juice of the spleen, liver, or kidneys of swine just dead from the disease or killed during its last stages. It is a very small, slender bacillus, only 1 to 1.5 micra long; it occurs singly and in small groups, sometimes in chains which are wavy or angular in outline. Such chains are particularly clearly seen in the endocardial verrucous deposits, which may contain the organisms in very large numbers. The bacillus stains with the ordinary watery anilin stains, and is Gram positive. The latter stain shows the bacilli particularly well in blood smears, or smears from the organs mentioned, in which it is found in larger numbers or in sections of tissues. The bacillus is not motile, possesses no flagella, and forms no spores.

Cultural Properties.—The organism grows well at room and at incubator temperatures. It grows well in a very characteristic manner in *gelatin* as first described by Loeffler, Schütz, and Schottelius. On gelatin plate cultures inoculated from spleen juice or blood, hazy, bluish-gray, racemose, cloudy spots appear on the second or third day; they are situated a little below the surface of the medium, and can only be seen with some difficulty if the plate is placed on a dark background. If stab cultures in gelatin are made from one of these colonies, small round colonies, with lines radiating toward the periphery, appear along the stab; these lines become divided and finally form a hairy or cloudy mass. After six to ten days the gelatin culture has a very characteristic appearance which has been likened to that of a test-tube brush. The surface of the gelatin remains free; no growth occurs on it. On a streak culture on gelatin slants the colonies are more like those on gelatin plates. Sometimes, however, the colonies form round whitish or yellowish-brown globular masses, without the appearance of brush-like extensions. On *agar* and *blood serum* the bacillus grows very scantily, but better when the tubes are kept in an atmosphere from which the oxygen has been removed by Buchner's pyrogallic acid method. The organism grows in *bouillon*, and first slightly clouds the medium; later a scanty grayish-white deposit is formed. It does not grow on *potatoes* kept aërobically, but it has occasionally been raised on potatoes kept in an oxygen-free atmosphere. Smears from artificial cultures show the organism singly, in pairs, and in short, wavy, or angular chains; involution forms are frequently formed.

Resistance.—The bacillus of swine erysipelas is more resistant than most pathogenic non-spore-forming bacteria. It can remain alive for one month dried out and kept in the incubator at 37° C.; in the dried condition it resists sunlight for twelve days; and sometimes, in moist condition, a temperature of 70° C. for fifteen minutes. According to Petri it has survived in infected pork after broiling it for two and one-half hours; boiling of the meat in water, however, promptly kills the bacillus, which can remain alive a long time in salted or smoked meat and in buried cadavers.[1]

Natural Infection.—Hogs are the only animals subject to natural infection with the bacillus of swine erysipelas. They are especially susceptible when they are from three to twelve months old; sucking pigs are not very susceptible, nor are animals older than one year. The latter have probably acquired immunity by a previous attack, which may have been so mild as to have escaped attention. Natural infection occurs through the skin or the intestines; infection through the lungs by inhalation has never been proved. It is believed that the disease is most commonly contracted through the intestines, because large numbers of bacilli are found in them and also because the affection often occurs extensively among animals kept in one place under the same conditions. Food and water contaminated with the feces of sick animals are a fruitful source of spreading the disease to large numbers of animals. The importation of the disease into previously uninfected territories takes place through sick animals or their products. Both Cornevin and Kitt have demonstrated the infective character of the feces of sick animals. Olt, Jensen, and Baumeister have shown the presence of erysipelas bacilli in the tonsils, intestines, and lymph glands of otherwise healthy hogs.

Artificial Inoculation.—Gray and white mice, pigeons, and rabbits are very susceptible to artificial inoculation with fresh cultures of the bacillus or with blood, juice from the organs, etc., of hogs sick with the disease or dead from it. If a mouse is inoculated subcutaneously with a small amount of infected material it becomes very sick after twenty-four hours, and generally dies within four days under repeated attacks of suffocation. This form of death is quite characteristic in mice infected with the bacillus of swine erysipelas. Field mice are not susceptible to the organism. Pigeons die on the third or fourth day after inoculation, and they likewise, though not to so marked a degree as mice, show respiratory difficulty before the fatal termination. If rabbits are inoculated cutaneously or subcutaneously at the ear, an erysipelas-like swelling and redness appears at the point of injection. The local process may disappear, or it may spread and lead to a general infection and death. Sometimes death does not occur until after a prolonged cachectic condition. If rabbits are inoculated

[1] In cadavers it has been found alive after 280 days.

intravenously they promptly die within three to six days from a general infection. Inoculation experiments on hogs or feeding of infected material, as shown by Kitt, Schütz, Schottelius, and Preisz, lead to the development of a typical attack of erysipelas with septicemia.

Protective Inoculations.—The first successful attempt in protecting experimental laboratory animals and hogs against erysipelas infection were made by Emmerich and Mastbaum; these were followed by Pasteur's protective inoculations with cultures of the bacillus attenuated for hogs by being repeatedly passed through rabbits. The modern methods now universally used were worked out almost simultaneously by Leclainche, Voges and Schütz, and Lorenz. These methods consist in injecting an immune serum of high value and a virulent culture of the bacillus of swine erysipelas, either mixed or simultaneously, in different places or at different times, *i. e.*, first the immune serum, then the culture. The immune serum is generally prepared in the horse, occasionally in cattle. Horses in order to furnish an immune serum of high value generally receive first intravenously 100 c.c. of a bouillon culture of virulent erysipelas bacilli. It is necessary to begin with a comparatively large dose because equines do not react to small doses of 5 to 50 c.c. of bouillon cultures. The doses are increased in intervals of eight to ten days to 150, 200, 250, 300, and even to 500 c.c. In this manner a serum of high value is generally obtained in about two months. When the reaction after the last injection has subsided the blood is drawn, collected, and treated in the usual aseptic manner and prepared for preservation by the addition of 0.5 per cent. carbolic acid. Kitt, Schubert, and Pretter have also prepared an antiserum in a similar manner in cattle. It is claimed by some that a mixture of horse and cattle serum, a so-called "*double serum,*" is more powerful in its effect than the horse serum alone, but this is denied by others. The antiserum must be tested before use. The best animals for this purpose are gray house mice. A good strong immune serum should, in a dose of 0.01 gram, protect a mouse of 15 grams against 0.1 gram of virulent bouillon culture of the erysipelas bacillus. An immune erysipelas serum from horses or cattle meeting with these requirements is used in practice as follows:

For Therapeutic Purposes in Hogs Sick with Erysipelas.—A hog weighing up to 100 pounds receives 10 c.c.; hogs from 100 to 250 pounds, 20 c.c.; hogs weighing over 250 pounds, 30 c.c. The injection may be made at any place of the body, but the base of the external ear, or, if larger amounts are used, the knee fold are generally preferred. The therapeutic value of the injection is the better the earlier in the course of the disease it is used.

For Protective Purposes by the Simultaneous Method.—As already stated, protective inoculation may be made by the simultaneous method, or with an interval between the serum and the culture

injection. The doses recommended for hogs of different sizes are the following:

For hogs up to 50 pounds	3 c.c. antiserum
For hogs from 50 to 100 pounds	5 c.c. antiserum
For hogs from 100 to 250 pounds	8 c.c. antiserum
For hogs from 150 to 200 pounds	10 c.c. antiserum
For hogs over 300 pounds	15 c.c. antiserum

The bouillon culture employed for injection must be young and virulent. It is used in doses of from 0.25 to 1 c.c., according to the weight of the animal, but between these limits very great accuracy in the dose is unnecessary. *In the simultaneous method the immune serum and the culture may be mixed, or they may be injected at the same time in two different spots.* When the serum is first injected alone the culture must be injected after three to five days. The passive immunization by the serum alone produces immunity for a very short period only, but the combined passive and active immunization protects the animals treated for a period variously estimated at from six to twelve months. The statistics from France, Germany, and Austria concerning the value of the combined protective inoculation of hogs against erysipelas are very favorable.

Transmission to Man.—Preisz and other authors have reported a few cases of persons who having handled hogs sick with erysipelas or their meat, contracted a slight erysipeloid lesion of the skin, transitory in character, and not leading to any grave general disturbances.

THE BACILLUS OF MOUSE SEPTICEMIA.

Koch, when studying wound infection diseases early in his career, discovered a very minute bacillus, very fatal to mice, which he called the Bacillus murisepticus, or the bacillus of mouse septicemia. After Loeffler had discovered the bacillus of swine erysipelas it was found that the latter and the bacillus of mouse septicemia were so similar in morphologic and cultural properties that it appeared reasonable to consider them as one species. The question, however, is not yet fully settled. It is discussed by Preisz, who has given considerable attention to this matter in his article on swine erysipelas.[1] Preisz's statements are to the following effect. The question whether anybody has succeeded in finding the bacillus of hog erysipelas in the outside world is intimately connected with the question whether the bacillus is identical with that of mouse septicemia. Koch found the bacillus in putrefying fluids, Loeffler observed an epidemic among his mice due to it, Johne cultivated it from putrid meat, and Preisz from putrid cattle blood.

The morphology and cultural properties of the Bacillus murisep-

[1] Kolle and Wassermann's Manual, vol. iii.

ticus and of the bacillus of swine erysipelas may, under certain circumstances, show the very greatest similarity, but they may also, under absolutely like circumstances, show more or less apparent differences. The mouse bacillus may appear in the blood of infected animals still finer than the erysipelas bacillus. They may both show marked differences as to cultural properties in gelatin stick cultures. The mouse bacillus may grow very much more rapidly than the erysipelas bacillus under absolutely the same conditions. These differences, however, do not necessarily mean that the organisms are two species. No investigator has yet succeeded in producing a typical erysipelas in swine by inoculation with the Bacillus murisepticus, but Luepke claims to have produced the mild form of swine erysipelas known in German as "Backsteinblattern" (brick-pox). Rabbits and hogs, according to Lorenz, can be immunized against the bacillus of erysipelas by inoculation with the Bacillus murisepticus.

From the above it appears that the question of the identity or non-identity of these two bacilli cannot yet be considered as satisfactorily settled.

QUESTIONS.

1. What are the other names for swine erysipelas?
2. What kind of a disease is it?
3. What is the cause of the disease? Who discovered it?
4. Describe the pathologic lesion in the acute form of the disease.
5. What is a glomerulonephritis?
6. What is an endocarditis verrucosa? Is it found in swine erysipelas?
7. What animals are susceptible to natural infection? How is the disease contracted?
8. Is the bacillus of swine erysipelas sometimes found in healthy hogs, and where?
9. What laboratory animals are susceptible to artificial infection?
10. Under what symptoms does a mouse die when inoculated with material containing the bacillus of swine erysipelas?
11. How do rabbits act after subcutaneous and after intravenous injection?
12. Describe the morphology and staining properties of the Bacillus rhusiopathiæ suis.
13. Describe the cultural propertics, particularly the appearance of a gelatin stick culture.
14. Discuss the resistance of the bacillus.
15. Who developed the modern methods of serum therapy against swine erysipelas?
16. How is the antiserum prepared from horses or cattle?
17. What is meant by a double erysipelas serum?
18. How is the serum tested as to its protective power?
19. In what doses is the immune serum used when it is employed as a curative?
20. How is protective inoculation practised? Describe the three different methods.
21. What kind of cultures are used in active immunization and in what doses?
22. What is the bacillus of mouse septicemia? Who discovered it?
23. What is the relation of the bacillus of mouse septicemia to that of swine erysipelas?
24. Is swine erysipelas ever transmitted to man?

CHAPTER XXVI.

GLANDERS BACILLUS.

Occurrence and Historical.—Glanders, malleus, malleosis, farcy, "Rotz" (German), "morve" (French), is a disease which has been known to mankind for a long time. The name glanders refers to the lymph-gland-like swelling and to the swelling of the lymph glands. The designation malleus, malleosis, morve, is derived from a Greek word meaning a bad disease, and the German word "Rotz" is a generally employed but rather vulgar designation for a mucopurulent discharge from the nose. It is claimed that the word malleus was introduced by the celebrated Greek philosopher and naturalist, Aristoteles. The contagious nature of the disease among horses was recognized as early as the fourth century after Christ, but this fact was afterward forgotten and not rediscovered until about 200 years ago.

This infectious, contagious disease is found particularly among equines, but it is also communicable to man, sheep, goats, camels, carnivora, etc. Laboratory animals, like guinea-pigs, rabbits, etc., are likewise very susceptible. It is caused by a microörganism known as the Bacillus mallei, first discovered in 1882 by Loeffler and Schütz. Cattle are absolutely immune to the Bacillus mallei; pigs are very slightly susceptible and can be infected only with difficulty. Domestic birds are likewise immune.

Glanders is very prevalent almost over the entire world. It is found in Europe, Asia, Africa, and America. It has been introduced into the Philippine Islands, but so far it has been excluded from Australia, including Tasmania and New Zealand.

Mode of Infection and Pathologic Changes.—The disease may take either an acute, a subacute, or a very chronic course, the latter extending over years, and in each case it leads to local inflammatory changes, with the formation of granulation tissue. The glanders bacillus frequently gains entrance through the upper respiratory passages, and first becomes localized in the nasal mucosa; but it also frequently enters through wounds and abrasions of the skin or epithelial covering. From the portal of entrance the glanders bacillus spreads along the lymphatics, where it multiplies; later it invades the lymph glands and finally the internal organs. The infection is spread from animal to animal by direct contact or by coughing, in consequence of which small expelled particles of morbid material coming from a diseased animal are inhaled by a healthy one. The infection is spread indirectly

through feed, bedding, harness, etc., which have been soiled with the discharge from glanderous ulcerations or suppurating foci.

Wherever, in natural infection, glanders bacilli have gained entrance into the body of a susceptible animal, they multiply locally and cause a cell necrosis. This is followed by an inflammatory reaction with hyperemia, transudation, and emigration of leukocytes. The inflammatory reaction, however, does not limit the infection but the bacilli continue to multiply and the necrotic processes spread and with them the inflammatory reaction. The infection is spread both by direct extension from the multiplying glanders bacilli and by transportation of bacilli to neighboring or more distant parts of the body through the agency of leukocytes which have taken them up but have not killed them. At these points they again multiply and give rise to the same lesions which they produced at the original place of entrance. The infection may also be spread in a glanderous animal by the discharge from the lesions flowing over a mucous membrane. In this manner the disease may spread from the nose to the trachea, bronchi, and lungs; or, in primary pulmonary cases, the mode of extension may be from the lungs outward.

The anatomical changes which glanders produces in the skin, mucous membranes, and various internal organs vary according to the location and the virulency and acuteness or chronicity of the process. According to Kitt, four anatomical types of lesions are distinguished, namely: The glanders nodule, the glanders abscess, and ulceration, the glanders induration, and the glanders infiltration.

Nodules.—The formation of the glanders nodule may be studied in the nose of the horse. Following the invasion and multiplication of the glanders bacillus a small, slightly elevated, grayish-white, transparent nodule, varying in size from a small shot to a pea, is formed as the result of the inflammatory cellular infiltration. In consequence of the necrosis in the interior of the nodule a small abscess cavity in its centre soon develops. When the necrosis extends outward far enough a loss of substance occurs at the highest point and an open ulcer is formed.

Ulcers.—The glanders ulcers are at first generally spherical, but they become irregular, either by an irregular extension of the necrosis at the periphery or by the confluence of neighboring spreading ulcers. The ulceration in glanders have an excavated, eaten-out appearance. They are surrounded by elevated margins and the ulcer is covered by a thin, greenish-white, seropurulent discharge, sometimes slightly stained with blood. If dried out, it covers the ulcer as a dirty grayish-brown crust. In the skin the glanders nodules may be as large or larger than a hazel nut and a distinctly palpable abscess may be felt before the outer portion of the abscess wall is broken through and an ulcer formed. When glanders nodules are first formed in the lungs, as in the pulmonary form of the disease, they are small, grayish-white, translucent nodules which somewhat resemble young tubercles. The

bronchial glands become studded with such nodules and later with abscesses. In the skin the glanders abscesses, before leading to external ulceration, contain a thin, greasy, yellowish or yellowish-red pus, occasionally mixed with a necrotic cell detritus.

Indurations.—The glanders indurations are frequently found in the nose, where they present themselves in two types. In the one there is a cicatricial formation, following an ulcerative process which has come more or less to a standstill; in the other in which the glanders virus may not have been of a very virulent type from the beginning, and may not have led to ulceration, there is instead little necrosis and much connective-tissue proliferation, with the formation of fibrous connective tissue. Bands and ridges, stellate and radiating masses, projecting slightly over the surface of the surrounding more healthy mucosa are formed, either secondarily after ulceration or primarily. Sometimes the masses, instead of being rather firm, have a softer character, and the purplish color of young granulation tissue.

Infiltrations.—The glanders infiltrations are found particularly in the lungs in the pulmonary type of the disease. In the horse this type assumes first the character of a bronchopneumonia. Small areas of consolidation with nodule formation appear. These areas are of an elastic consistency and a gelatinous yellowish color, sometimes mixed with red or light brown. As they increase in age these areas spread and become confluent, forming larger sarcoma-like masses, which when incised are either homogeneous and grayish red or white in color, or they may be honeycombed with smaller or larger abscess cavities, filled with pus and a cheesy or moist chalk-like granular material. Associated with this condition in the lungs is an enlargement and hyperplastic inflammation and purulent cavity formation in the thoracic lymph glands. A diffuse, gelatinous infiltration is formed in the lymph channels. Such infiltrations of a gelatinous character are also frequently found in the lymph channels of the skin in skin glanders.

Pulmonary Glanders.—Pulmonary glanders in the horse, as already stated, generally first assumes the type of a lobular or bronchopneumonia, no matter what the sequel may be. In the cat tribe it generally begins with the consolidation of an entire or of several lobes, and has anatomically the character of a lobar, fibrinous, or croupous pneumonia. According to McFadyean the lungs always sooner or later become involved in glanders, wherever the original portal of entrance may have been.

Microscopic Changes.—These vary somewhat in the acute and chronic type. In the acute type a central area of marked necrosis with cell degeneration and nuclear fragmentation is generally found. Around the necrotic area many polynuclear leukocytes are seen; many of them are themselves degenerated, others intact. The vessels in the inflamed but not yet necrotic area are greatly congested. In the pulmonary cases *in the horse,* multinuclear giant cells are frequently

seen in the inflammatory zone next to the necrotic area. In the slow chronic cases the histologic examination shows the presence of much fibrous connective tissue.

Susceptibility.—That man is not as susceptible to the natural glanders infection as the solidipeds, can be seen in the fact that many men handle affected horses, and yet, on the whole, the number of persons contracting the disease is comparatively small. On the other hand, the number of scientific investigators working with the Bacillus mallei in the laboratory and contracting a fatal infection has been alarmingly large. *Hence, students working with live glanders*

FIG. 147

The pustular eruption of acute glanders in man as exhibited on the day of the patient's death, twenty-eight days after the initial chill. (Zeit.)

cultures should be exceedingly careful. The first infection of glanders in man was recognized by Lorin in 1812. By far the greatest number of cases of natural glanders infection in human beings occurs among hostlers, drivers, farmers, horse butchers, and other habitual handlers of horses. In these trades the disease is generally contracted through abrasions or wounds of the skin, commonly leading to the formation of a pustular eruption which has very frequently been mistaken for smallpox and also for a gangrenous erysipelas. In laboratory workers the disease generally begins as a respiratory (nasal and pulmonary) infection. Even this mode of infection, however, is likely to lead to a pustu-

lar, cutaneous eruption before death occurs. In man the various types usually take an acute rapid course. The chronic type of cutaneous glanders with the formation of abscesses, ulcers, and lymphatic swelling and involvement has also been observed in man. Cases of this kind have formerly been mistaken for tertiary syphilitic lesions. More acute cases resembling smallpox in man have been reported by Wherry, Zeit, Bevan and Hamburger, and others.

Morphology.—The Bacillus mallei varies considerably in size and shape according to the culture medium on which it has been grown. It is, as a rule, rather slender; occasionally, however, it is short and plump. It is from 2 to 5 micra long and from 0.5 to 1 micron in diameter. On old potato cultures the bacillus sometimes appears in long filaments forming intertwined, irregular masses. Bacilli of the ordinary, most common type are generally not perfectly straight, but slightly curved. They do not stain uniformly, but somewhat in the manner of the diphtheria bacillus. The Bacillus mallei is not actively motile, but shows a very lively molecular movement; it does not form spores nor does it stain well with the ordinary watery anilin stains, but takes better the stronger stains such as Loeffler's alkaline methylene blue, Kuehne's carbolmethylene blue or carbol-thionin. Loeffler has recommended the following method for staining for glanders bacilli in smears from suspected pus or necrotic material:

1. Prepare the cover-glass smear in the usual manner, air dry, fix and float the cover-glass for five minutes on Loeffler's alkaline methylene blue.

2. Dip for one second into a 1 per cent. watery solution of acetic acid to which enough of a watery solution of tropeolin 00 has been added to give it a Rhine-wine yellow color.

This last step decolorizes the cell protoplasm entirely and the nuclei partially, so that the deep blue stained bacilli can be more readily found.

Cultural and Biologic Properties.—The Bacillus mallei can be obtained in pure culture without great difficulty. It is generally impossible to obtain it directly from the discharges from glanderous lesions in the horse. As a rule, it is necessary first to inoculate a guinea-pig in the manner described below. The bacillus grows best between 30° to 40° C.; growth ceases at and below 20° C. and above 43° C. It is a strict parasite which has never been found under natural conditions except in connection with cases of glanders. If cultures have been raised for many generations on artificial media the bacillus may also grow at temperatures lower than 20° C. The organism grows much better in artificial cultures in the presence of oxygen; in its absence there is only a very poor development. The culture media may be faintly alkaline, neutral or faintly acid; the latter reaction is most favorable. The addition of glycerin, 4 or 5 per cent. to the agar or bouillon, is advantageous to the development of the Bacillus mallei. In ordinary *bouillon* or glycerin bouillon the Bacillus mallei

grows rapidly, and the medium after twenty-four hours shows a slight uniform clouding; later a whitish, slimy sediment is formed without clearing of the supernatent fluid. If the fluid is not disturbed a whitish, slimy surface pellicle is also formed, and even after this has sunk to the bottom a white ring or margin remains at the circumference of the surface and the glass. On slightly acid glycerin agar the organism develops well, but there is nothing characteristic about the growth. The colonies are first flat, dull white or grayish, translucent; later they become somewhat yellowish or perhaps even somewhat reddish yellow. The colonies rapidly become confluent in the incubator, slowly at lower room temperatures. The organism grows well on horses' and sheep's blood *serum*, and not so well on cattle blood serum. The colonies after about three days appear as yellowish, translucent drops on the surface of the coagulated serum. They possess a tenacious, slimy, viscid consistency. After eight to ten days the growth on blood serum becomes opaque and grayish white. On *potatoes* the growth of the Bacillus mallei is most characteristic; it appears after about two days as a delicate, yellowish, translucent cover, and on the third day assumes an amber color. After six to eight days the growth has become quite abundant. It is now opaque and has lost most of its transparency, being reddish yellow in color. The surface of the potato not covered by the growth has assumed a faintly green hue. This zone, however, may not be very noticeable or it may be yellowish green or brownish green. In using potatoes for the cultivation of the Bacillus mallei it is important to select such as are not too acid, or, still better, to correct the acidity before sterilization. Potatoes which have been frozen or which have begun to germinate should not be used, because they are likely to contain sugar from which the Bacillus mallei forms acids. It is best to immerse the disks or pieces of potato, after washing and peeling, for one hour in a 0.5 to 0.7 per cent. solution of bicarbonate of soda and then to sterilize them. In this manner a material of not too high acidity is obtained. Certain other bacteria when growing on potato form a growth more or less similar to that of the Bacillus mallei. The most important of these is the *Bacillus pyocyaneus*. This organism produces a yellowish-brown growth, which, however, is not transparent; older colonies also exhibit a mother-of-pearl luster not shown by the Bacillus mallei. A simple test to differentiate between the two organisms when grown on potatoes and closely resembling each other is to spread some of the growth on a piece of filter paper and expose it to ammonia vapor. If the growth is Bacillus pyocyaneus a characteristic *bluish-green color* at once appears; this is *not* shown when the growth is Bacillus mallei. An evidently rare pseudomalleus bacillus, discovered by Babes, forms a growth like the genuine glanders bacillus on potatoes, and can be accurately differentiated only by animal experiments. In guinea-pigs, field mice, and cats it produces local processes only, but kills rabbits.

Resistance.—The Bacillus mallei may be kept alive for a long time in artificial cultures, provided these are sealed and kept in a cool, dark place. The bacillus is easily killed by heat, an exposure for ten minutes at 56° C. destroys it. It is likewise easily killed by the ordinary antiseptics, such as corrosive sublimate, carbolic acid, etc.

Bose reported that he exposed pieces of cotton, etc., infected with young, virulent glanders bacilli to the action of formalin vapors and that they were killed after five hours. The author, however, has found that young cultures on glycerin agar tubes, exposed after the removal of the cotton plugs in a tightly closed anatomical jar to formalin vapors, survived after three days. It appears, accordingly, that formalin vapors are not to be depended upon in disinfecting stables where cases of glanders have occurred. Sulphuric acid in ½ to 2 per cent. solutions, milk of lime, chloride of lime, and the chemicals of the carbolic acid group are dependable disinfectants for glanders-infected buildings, harness, etc.

Diagnosis.—In typical advanced cases the diagnosis of glanders may be made from the clinical findings. As a rule, an accurate diagnosis cannot be made from the microscopic examination of a nasal or cutaneous purulent discharge, but only after inoculating a guinea-pig or after the mallein test or the agglutination test.

The microscopic examination of virulent discharges is much invalidated by the fact that they generally contain a mixture of bacteria among which it is difficult and often impossible to recognize the glanders bacillus. The probability of a successful microscopic diagnosis is much greater when the material for examination consists of soft necrotic material from the interior of a not yet ulcerated or opened glanders nodule, either from the nasal mucosa, the skin or a submaxillary gland. The necrotic or purulent material is spread on a cover-glass, air dried, fixed, and stained with Kühne's carbol methylene blue or with Loeffler's methylene blue, and then washed, as already described, in an acetic-acid tropeolin 00 watery solution. Even in favorable material glanders bacilli are present only in moderate numbers, but a diagnosis may be obtained if they are characteristic in shape and staining properties (Gram negative) and other bacteria are absent. The attempt may also be made to raise pure cultures from such material obtained aseptically and showing only one kind of bacillus.

The Biologic Test for Glanders (Strauss' Test).—The best animal for the inoculation test for glanders is the male guinea-pig. The test is made as follows: Some of the suspected material obtained under very aseptic precautions is rubbed up with sterile physiologic salt solution and 1 to 2 c.c. of the mixture is injected with a sterile hypodermic syringe into the peritoneal cavity of the animal. The injection is made immediately above the symphysis pubis. When the material contains virulent glanders bacilli a swelling of the testicles, which are now hot and painful, develops after two to four days. If the animal

is undisturbed the testicles later ulcerate, break through the skin, and discharges a purulent fluid containing many bacilli. The animal becomes emaciated and dies after ten to twelve days. The post-mortem examination shows numerous glanders abscesses in the testicles, the lymph nodes and in the internal organs, such as the liver, spleen, kidneys, lungs, etc. When the test, however, is made for diagnostic purposes the animal is not allowed to die, but is killed as soon as the testicle shows a marked inflammatory swelling, which is generally the case after two to three days and a postmortem examination is made. In the case of glanders the examination of the testicles shows small grayish-white transparent nodules, sometimes even small abscesses, and smears from the nodules and abscesses show the typical glanders bacilli. If the testicles are opened under aseptic precautions the glanders nodule can also be used to obtain pure cultures of the Bacillus mallei. It is always necessary to examine smears from the swollen, inflamed testicles of the guinea-pig microscopically and to stain with Loeffler's or with Kühne's stains and by Gram's method, because certain other bacteria also bring about an orchitis (inflammation of the testicles) if inoculated intraperitoneally into a male guinea-pig. These bacteria, however, are all Gram positive. They are Nocard's bacillus of ulcerative lymphangitis of the horse. Preisz's bacillus of pseudotuberculosis of the sheep, Kutscher's bacillus found once in the nasal secretion of a horse, and also sometimes the Bacillus pyocyaneus. Young cats may also be used for subcutaneous inoculation with glanders-suspected material. They develop first a local swelling and ulceration and later a general glanders infection.

The Mallein Test.—Mallein[1] is a vaccine, prepared from killed, virulent glanders bacilli. It is injected into a soliped suspected of glanders, and if the disease is present a typical local and general reaction occurs. Very little is known concerning the mode of action of the Bacillus mallei, but there is good reason to believe that its pathogenic properties depend, if not exclusively, at least largely, upon resistant endotoxins. The latter are present in the mallein; they represent the antigen (see chapter on Antibodies), which gives rise to the formation of specific glanders antibodies. The local and general reaction in malleus-infected horses, when injected with mallein, is probably due to an interaction between the malleus antigen and the malleus antibodies.

Mallein is prepared according to various methods; that of Roux, of the Paris Pasteur Institute, is as follows: The virulent cultures of glanders bacilli are obtained by long-continued intravenous injections of bacilli into rabbits. When the cultures have become so virulent that they will kill rabbits in thirty hours they are grown in flasks

[1] The term *mallein* was formed on the analogy of the word tuberculin. The term was first used by Helman, the original maker of the vaccine. German writers sometimes use the term "*Rotzlymphe*," and French authors the word "*morvin*," for mallein.

containing 250 c.c. glycerin bouillon. After one month's incubation at 35° C. these cultures are sterilized in the autoclave under atmospheric pressure at 100° to 115° C. They are then evaporated down on a water bath to one-tenth of their original volume and filtered through a particular filter paper. The concentrated end-product of these procedures is known as the *raw mallein*, or *"mallein brute."* It is a dark brown, syrupy fluid containing 50 per cent. glycerin, and it can be kept in corked bottles. Before use this concentrated mallein must be diluted with a one-half per cent. watery solution of carbolic acid. The dose of the dilute mallein for a horse is 2.5 c.c. The injection is made either in front on the thorax or on one side of the neck under aseptic precautions and with a sterile syringe. It is best to shave closely the place where the injection is made. Before making the mallein test the temperature of the animal should be taken three times a day, morning, noon, and evening, for two days. Horses with fever do not give accurate results with the mallein test. After the injection is made the animal should be kept quiet in a well-protected stable, with fairly even temperature; it should not be overfed nor permitted to drink large amounts of cold water. From six to sixteen hours after the injection the temperature is to be taken every hour, and from then to the thirty-sixth hour to the forty-second hour every two hours, with the exception of a night's omission, for about eight hours. These strict rules cannot always be observed, but the temperatures should be taken at least as follows: Three times before the injection; then every two hours for six to twenty hours after the injection. It is well to give the injection very late at night, so that the taking of the temperature may be begun every two hours very early the next morning.

Effect of the Mallein Test upon a Horse not Having Glanders.— The temperature frequently rises after a few hours, but rarely reaches 40° C., and goes down to normal a few hours afterward. Locally there may be very little reaction, or there may be a slight painless edema, which disappears within twenty-four hours after the injection.

Effect of the Mallein Test upon a Horse Affected with Glanders.— The temperature begins to rise six to eight hours after the injection; the curve then rises rapidly during the next six to eight hours, and reaches its maximum with 40° to 42° C. This maximum is kept with some slight variations for another eight hours, and then the temperature gradually sinks. Some elevation of temperature generally remains twenty-four hours after the injection. On the second day a similar rise of temperature occurs; as a rule it is not as intense as on the first day, but occasionally it is more so. The rise in temperature sometimes appears soon after the injection (1 hour); sometimes it is considerably delayed (twenty-two hours). The *local reaction* at the site of the injection is very characteristic. It makes its appearance in six to eight hours and consists in a very painful, well circumscribed, rather firm, doughy hot swelling about four to six inches in diameter. Later the boundaries of the swelling become more diffuse and less

sharply defined. The pain generally ceases or becomes less marked on the second day, but the infiltration spreads, often to the extent of ten inches or even more in diameter. Only after three to eight days does the last trace of the swelling disappear. The other symptoms, such as malaise, loss of appetite, weakness, are inconstant and depend largely upon the height of the fever and upon individuality. Some horses infected with glanders become very irritable and sick after the injection of a full dose of mallein. The dose varies in the different preparations, and is generally unmistakably indicated on the label. It is, of course, easy to distinguish between an absolutely negative and a typical fully developed positive reaction, but cases occur in practice in which the decision is difficult. Horses very much advanced in glanders sometimes do not react typically, but in such cases a mallein test is generally unnecessary as the diagnosis can be made from the clinical symptoms.

The Eighth International Veterinary Congress, Buda-Pesth, 1905, adopted the following rules for the mallein test and its interpretation:

"1. Unless the results following the injection of mallein exhibit the characteristics of a typical reaction they must not be regarded as indicating the existence of glanders.

"2. A typical reaction comprises a rise in temperature of at least 2° C. The rise should extend above 40° C. (104° F.). During the course of the first day the temperature curve usually exhibits a plateau of two peaks, and on the second, and even on the third, a more or less marked rise. This rise in temperature is accompanied by a local and general reaction.

"3. Any rise in temperature which falls short of 40° C. (104° F.), and higher atypical reactions, necessitates a second test.

"4. A gradual rise of temperature sustained for some time is indicative of glanders, even though it differs from the ordinary type of diagnostic reaction.

"5. The local typical infiltration at the point of injection is a certain indication of glanders, even when rise in temperature and the general organic reaction fail.

"6. Animals which have undergone the mallein test, whether or not without reaction, should always be tested a second time after an interval of ten to twenty days.

"7. The preparation of mallein should only be intrusted to scientific Government institutions, or to institutions recognized and controlled by the State.

"8. With the object of determining the full value of mallein, and of clearing the many still unexplained points in regard to the mallein reaction, the Congress requests the various European Governments to appoint committees to study the question."

In testing mules double doses of mallein (2×2.5 c.c. = 5 c.c.) should be used; also in retesting after an interval of a few weeks in doubtful cases.

Choromausky has recommended a so-called ophthalmo test for glanders. It consists in the instillation of a very small amount of mallein into the conjunctival sac of the eye. According to Wladimiroff this reaction is not very accurate, since horses free from glanders may also react positively by an inflammatory hyperemia of the conjunctiva.

Agglutination Test.—If the blood serum of horses suffering from glanders is allowed to act on a young, uniformly cloudy bouillon culture of glanders bacilli the latter becomes agglutinated, form small lumps, and these fall to the bottom of the test-tube or the watch-glass in which the test is made. (McFadyean.) This change is due to the precipitins and agglutinins present in the blood serum of glanderous horses. Such substances, however, are also present in the blood of healthy horses, but in much smaller amounts, and for this reason the agglutination test in glanders has to be made as a quantitative test. As a rule, serum from a healthy horse will agglutinate in a strength of 1 in 200 to 300 or even 400; while blood serum from a glandered horse will agglutinate the bacilli in a dilution of 1 in 1000, 1500, and even more.

From a large number of tests made on healthy animals and on horses suffering from glanders the following rules may be drawn:

Horses whose blood agglutinates only in dilution up to 500 are probably healthy, yet among them occur a few cases (about 6 per cent.) of glanders.

Horses agglutinating in a dilution up to 800 are probably affected with glanders, yet about 3 per cent. of them are free from this disease.

Horses whose blood serum agglutinates in dilutions up to 1000 or more are surely infected with glanders.

Parke, Davis & Company have devised an apparatus called by them the *Glanders Agglutometer*, which very much simplifies the agglutination test for the veterinary practitioner. It includes an emulsion of killed glanders bacilli and circular pieces of filter paper varying in size for procuring a fairly definite amount of serum. Various sizes of filter paper are dipped into the serum to be tested, and are then placed in small test-tubes, which are filled up to a mark with the bacterial emulsion. In this manner dilutions of 1 in 200, 1 in 500, 1 in 800, and 1 in 1200 are obtained. The tubes are set aside in a warm place for a number of hours and then inspected. It is noted in what dilutions agglutination and precipitation has occurred.

Whenever an agglutination test is made it is necessary to draw some blood from the suspected horse; this is best done from the jugular vein, with a sterile syringe. The blood may then be allowed to coagulate spontaneously, and after several hours the serum can be removed, or the latter may be separated and collected at once by the aid of a centrifuge.

The Value of Mallein as a Diagnostic and as a Curative.—The value of mallein as a diagnostic of latent and occult glanders in equines has been established beyond doubt. It has become the most important

agency in the campaign to stamp out glanders and to protect healthy animals against the danger of infection from sick ones. If all the horses of a stable, company, or landowner are at intervals subjected to the mallein test, immediately upon acquisition and a few times thereafter at periods of six to twelve months, glanders can be entirely suppressed and kept out. Before the general use of the mallein test this was impossible, because the disease often takes a very chronic and latent course, and a number of animals may be infected from a single sick one before the disease can be safely detected by manifest clinical symptoms. Since the glanders bacillus is a strict parasite and cannot exist for any great period external to the body of susceptible beings, the stamping out of the disease by united, systematic and rational efforts, should be accomplished within a short time. Whether mallein used in repeated, increasing doses, as recommended by Babes, Pilavios, MacFadyean, has really a curative effect in glanders is a question which has not yet been settled definitely, though Nocard and others have reported a number of such cases in which a cure evidently followed the systematic use of mallein.

PSEUDOGLANDERS.

Several other infections in horses not only clinically, strongly simulate glanders, but the causative microörganisms are pathogenic to guinea-pigs and act very much like the glanders bacillus, so that an erroneous diagnosis is easily possible unless the mallein test is employed. One of these diseases in horses is known as *lymphangitis ulcerosa* (*pseudofarcinosa*). This disease, comparatively common in France, has been studied extensively by Nocard, who, in 1892, discovered as its specific cause a bacillus now known under the name of Bacillus lymphangitidis ulcerosa. Clinically the disease is characterized by cutaneous abscess formation, suppurative ulcers, and swelling of subcutaneous lymph glands, a picture resembling skin glanders or farcy. The lesions do not remain localized in the skin or in the subcutaneous connective-tissue lymph glands, but deeper glands become involved, particularly those of the inguinal region, those along the seminal cord, those of the perineal region, and finally the kidneys themselves become the seat of abscesses. In fatal cases the lungs also show metastatic abscesses.

The bacillus of ulcerative lymphangitis in the horse is found in large numbers in the pus. It is rather a plump and short rod, with rounded ends, often ovoid and broader in the middle, also sometimes club-shaped. *It is Gram positive.* It can be cultivated best in the incubator at 30° to 40° C., and does not grow at room temperature. In *nutrient bouillon* it forms, after three days, a whitish granular sediment, while the supernatant fluid becomes clear. A delicate pellicle is sometimes formed on the surface. The growth in *glycerin*

bouillon is quite abundant. On *agar* small, whitish, opaque colonies round or wavy in outline, are formed. These, after a few days, become confluent and cover the medium with a delicate, moist, easily detachable growth. On *potatoes* a scanty, dirty grayish, dry, dusty growth occurs. The best medium is *coagulated blood serum* on which small, round, distinctly margined colonies, elevated at the centre, are formed. These colonies later form racemose processes. On *horse's serum* the color is whitish, on *cattle serum* more yellowish, sometimes as intense in color as colonies of the Staphylococcus pyogenes aureus. The organism is *strictly aërobic.* It does not change the reaction of the medium and does not grow well in *milk,* which it does not coagulate. Cultures remain virulent for three or four months. The bacteria are killed in less than fifteen minutes at 65° C. and in one hour at 58° C. Nocard was able to produce the typical disease in two horses by the subcutaneous inoculation of two drops of a culture. Guinea-pigs inoculated subcutaneously develops large abscesses in four to five days; these heal slowly with scar formation while new abscesses develop in the neighborhood.

A small amount of pure culture or pus from a horse inoculated into the peritoneal cavity of a male guinea-pig produces an orchitis similar to that produced by the inoculation of glanders bacilli. Between the third and the fifth day the scrotum becomes inflamed, edematous, hot, tender, and painful. Death occurs after six to eight days. Sometimes the orchitis is relatively moderate, and death does not occur. The testicles may become entirely destroyed by the necrotic, suppurative process. Rabbits survive after intraperitoneal injection; subcutaneous injections produce an erysipeloid reaction. These animals die in a cachectic condition after intravenous injection. Mice and pigeons are susceptible, chickens are not.

Another bacterium found in the nasal discharges of horses, which when inoculated intraperitoneally into male guinea-pigs produces an orchitis and kills the animals in four to five days, is a bacillus discovered by Kutscher. This organism is also fatal to mice when inoculated subcutaneously. These bacilli, morphologically, are very much like the Bacillus mallei, but they are *Gram positive,* while the glanders bacillus is Gram negative. The bacillus of Kutscher also differs from the Bacillus mallei in that it *liquefies gelatin.* On *blood serum* it forms a deep orange pigment and on potatoes a thin, gray, dry growth.

Pseudofarcy due to a microörganism of the *saccharomyces* or *blastomyces* type is discussed in a later chapter.

QUESTIONS.

1. What are the other names for equine glanders?
2. What does malleus or morve mean? What "Rotz"?
3. What animals are naturally susceptible to the disease? What form of glanders is found in cattle?
4. What microörganism is the cause of glanders? Who discovered it?
5. Where is the disease found?
6. Give mode of infection and spread of glanders in an infected animal.
7. What type of lesions are produced in the tissues by the glanders bacillus? (Describe the first effects in detail.)
8. What are the four anatomical types of glanders lesions in the horse?
9. Describe them in detail.
10. Describe the pathologic changes in pulmonary glanders in the horse.
11. Discuss glanders infection in man. What other human diseases may it be confounded with?
12. Describe the morphology and staining properties of the Bacillus mallei.
13. Describe Loeffler's method of staining glanders bacilli in smears from pus, necrotic material, etc.
14. How is a pure culture of the glanders bacillus generally obtained?
15. Describe its cultural properties.
16. On what culture medium is the Bacillus mallei growth most characteristic? What are the characteristic features?
17. Describe the preparation of potatoes as a culture medium for the Bacillus mallei.
18. What is the pseudomalleus bacillus of Babes?
19. Discuss the resistance of the glanders bacillus.
20. Why is the microscopic diagnosis of glanders from simple cover-glass preparations inaccurate?
21. Describe Strauss' biologic test for glanders, and state what is found when the test is positive.
22. What is mallein? Describe its preparation.
23. Describe in detail the mallein test for glanders.
24. Describe its result (*a*) in a horse free from glanders; (*b*) in an animal suffering from the disease.
25. Is there any danger in subjecting a horse to the mallein test?
26. What is an ophthalmo test? What is the value of this test for glanders in horses?
27. Discuss the principle of the agglutination test in glanders. Describe its steps and the result in healthy and in glanders-sick horses.
28. Discuss the diagnostic value of the mallein test.
29. What is the value of mallein as a bacterine or vaccine in the curative treatment of glanders? Is glanders always a progressive and fatal disease?
30. Name an infection in horses which simulates glanders and in which the bacillus causing it gives a positive reaction in Strauss' biologic test on male guinea-pigs?
31. Describe Nocard's Bacillus lymphangitidis ulcerosa and point out particularly the features in which it differs from the Bacillus mallei.
32. What is Kutscher's pseudoglanders bacillus? How does it affect a male guinea-pig when injected intraperitoneally? What are the two most important features which differentiate Kutscher's bacillus from the Bacillus mallei?

CHAPTER XXVII.

BACILLUS OF INFECTIOUS ABORTION—STREPTOCOCCUS IN ABORTION IN MARES—STREPTOCOCCUS OF VAGINITIS VERRUCOSA OF CATTLE.

BACILLUS OF INFECTIOUS ABORTION.

Occurrence and Historical.—Infectious abortion, abortus enzoöticus "Seuchenhaftes Verwerfen" (German), "avortement epizoötique" (French), is the name given to that form of abortion in cows not due to accident or external influences or to ergot contaminated fodder, but to a specific microörganism known as the bacillus of infectious abortion of Bang. That this form of abortion in cows is infectious in character was recognized more than a hundred years ago in England. Frank, Lehnert, and Braeuer, between 1876 and 1880, proved this contention by infecting healthy cows from the vaginal discharges of cows which had aborted. Nocard, in 1885, studied the anatomic changes of the disease, while Bang, assisted by Stribolt, discovered the specific bacillus in 1896. Its etiologic relationship to the disease was confirmed by Preisz in 1902. Infectious abortion in cows has been observed in various countries in Europe, also in the United States. It was studied by Chester, Law and Moore, who failed to find Bang's bacillus, but instead an organism which appeared to belong to the colon-hog-cholera group of bacteria.

Pathologic Lesions.—According to Bang's findings, made on material very favorable for the study of the affection, the external serous surface of the uterus is normal, the cervical canal closed by tenacious mucus. An abundant, not fetid, exudate, consisting of a dirty yellowish, thin, mushy material, containing lumpy, mucoid masses and presenting in some portions where most of the fluid had been absorbed a semisolid character, is present between the uterine mucosa and the ovum. The exudate is alkaline in reaction. After its collection in a conical glass it separates into two layers, an upper composed of a reddish-yellow cloudy serum and a lower one of a thick, dirty, grayish sediment. The chorion upon section presents on its inner surface a gelatinous substance in which thin membranes are found. This layer is the delicate connective tissue between chorion and allantois in a condition of edematous infiltration. The latter extends to the fetus and spreads into it to the depth of about 1.5 cm. and also into the umbilical cord. The allantois and amniotic fluids are normal. The exudate between the uterine mucosa and the

fetal membranes contains the specific bacillus of Bang in enormous numbers.

Natural Infection.—This is brought about by the bull, who spreads the disease from infected to healthy cows. It is also believed that the infection may be spread by contaminated straw, bedding, etc.

Artificial Inoculation.—Pure cultures of the bacillus inoculated into the vagina and intravenously, as practised by Bang, caused abortion in cows and sheep and in a mare.

Morphology.—If a cover-glass preparation is made from the exudate between the uterine mucosa and the fetal membranes very characteristic bacteria are found in great numbers. Many of them are free between cells, many others are found in cells in such masses that the cell bodies are much swollen and extended. These intracellular bacteria are sometimes so dense that the cell itself has become unrecognizable; at other times, however, the nucleus can still be demonstrated. The organisms on first sight appear like cocci, but a more careful exaimnation shows them to be small, short, unequally staining bacilli. Some of them are as large as the tubercle bacillus, others are quite short and coccus-like. The unequal staining depends upon the presence of one, two, or three deeper staining granules. The organism is Gram negative.

Cultural Properties.—Pure cultures of the bacillus can be obtained on a special medium devised by Stribolt, which is prepared as follows: The basis is formed by the ordinary slightly alkaline nutrient bouillon to which $\frac{3}{4}$ per cent. agar has been added. This medium is then filtered clear and 5 per cent. gelatin is added. The reaction is again corrected, and after a clear product has been obtained it is distributed to test-tubes which are sterilized by the fractional method and finally cooled down to 45° C., when about one-half the quantity of sterile fluid blood serum (also warmed to 45° C.) is added. A tube is then inoculated from the material containing the organisms and dilutions to other tubes, are made in the usual manner. Tube No. 2 is inoculated three times from tube No. 1 with a sterile platinum loop, tube No. 3 in the same manner from tube No. 2, etc. The inoculated tubes are then rapidly cooled in cold water. When tubes so inoculated are kept in the incubator at blood temperature very small punctiform or sometimes slightly larger round colonies are formed. These are situated about $\frac{1}{2}$ cm. below the upper surface and extend from 1 to 1.5 cm. down. Thus growth takes place in a very definite zone, but neither above nor below it. The bacillus does not grow in *gelatin-agar;* it grows very scantily in a 5 per cent. glycerin bouillon, but better in a mixture of two parts of such a glycerin bouillon to which one part of serum has been added. In fluid media a granular sediment is formed after several days. The relation of the bacillus to oxygen is peculiar. It does not grow under strictly anaërobic conditions. It grows better in *glycerin bouillon* if pure oxygen is conducted through the fluid, and if afterward the opening of the tubes or flasks are sealed air-tight

with paraffin. While the bacillus in ordinary air does not grow up to the surface of the solid culture media, the presence of an atmosphere rich in oxygen favors growth and makes it much more abundant. This is true even when an excess of carbon dioxide of from 4 to 5 per cent. is present. Bang found two optima of growth, one in the presence of atmospheric air, and one in the presence of almost 100 per cent. oxygen. If the air above the solid culture medium is rarefied to a certain degree the growth reaches to the surface; if the rarefication becomes excessive, growth ceases entirely.

MacNeal and Kerr have recently found the bacillus of contagious abortion in some cases of abortion in cows in Illinois. In order to isolate this peculiar microörganism, they have made use of a new plate or Petri dish method devised by Nowak, which is as follows: Ordinary agar is melted and cooled to 50° C.; then mixed with about one-fourth its volume of naturally sterile blood serum, and poured into sterile Petri dishes, where it is allowed to solidify. The piece of placenta or other material from the abortion is streaked over a number of Petri dishes in the manner generally employed in preparing streak dilution cultures. The plates are then incubated for twenty-four hours at 37° C., to allow contaminating aërobic bacteria to develop. Colonies which have developed after twenty-four hours are marked with a ring on the glass by the aid of a glass pencil, India ink, or a cut-out label etc. The plates are then put into an anatomic jar, Novy jar, or desiccator, with a culture of the Bacillus subtilis. About one square centimeter of subtilis culture is to be used for each 15 c.c. of air volume of the jar. The jar is then closed and kept in the incubator for three days. The growth of the subtilis bacillus is used to absorb some of the oxygen of the air in the jar and to bring about those conditions which favor the growth of the abortion bacillus. If any of these are present they develop as transparent colonies with the characteristics already described.

Resistance.—The bacillus dies out rapidly in pure cultures (in about two weeks). It is killed at 55° C. in three minutes, in corrosive sublimate 1 to 2000 in fifteen seconds, in 1 per cent. carbolic acid in one minute, in 2 per cent. acetic acid in two minutes, and in 1 per cent. acetic acid in twenty minutes. It can remain alive and virulent in dried uterine exudate where it had its natural habitat, in the uterine cavity, and in dead embryos for many months.

Diagnosis.—McFadyean and Stockman have recently studied the distribution and diagnosis of epizoötic abortion in Great Britain. They ascertained the existence of the disease in fifty-five farms, distributed over thirty-six counties. They examined 51 feti and found the bacillus discovered by Bang in 22 feti. In 35 fetal membranes they obtained the bacillus in 33 specimens, while they failed in only 2; hence they consider the examination of the membranes for the presence of the Bang bacillus a more trustworthy procedure than the bacterial examination of the fetus. The two investigators attempted

to work out an accurate method of diagnosis by means of *vaccine* or *serum tests*. An *agglutination* test was devised, based upon the same principle and technique as the agglutination test for glanders. It was found, however, that the difference between the agglutinating powers of the serum of a cow affected with abortion and that of normal animals was too slight to inspire confidence in such a test. It was found during these experiments that the milk of an animal which had aborted possessed agglutinating properties up to 1 to 25, but owing to the opacity caused by the addition of milk to a culture the lacteal fluid was found unsuitable for such agglutination tests. Another test tried was one based upon the principle of *complement fixation*, which has found such wide application in the diagnosis of latent human syphilis (see Chapter VII). The serum for this test for epizoötic abortion is obtained from blood drawn from the jugular veins of the cows. The antigen is prepared from a pure culture of the abortion bacillus emulsified in physiologic salt solution. The complement is derived from the serum of guinea-pig's blood; the hemolytic amboceptor from the serum of goats sensitized for ox-blood corpuscles, and the latter were, of course, used for the final hemolytic test. As controls the blood serum of healthy cows and of cows infected artificially with the abortion bacillus were used. Unfortunately, the results of these complicated and painstaking tests of the authors, the first to apply the principle of complement fixation to the diagnosis of an animal disease, were not uniform and trustworthy. The last series of experiments for the diagnosis of epizoötic abortion was made with a *vaccine* prepared according to the principles which underly the preparation of tuberculin and mallein. This *abortin*, derived from pure cultures of the abortion bacillus, was injected, and the following conclusions as to its value as a diagnostic were drawn: "It would appear that a rise of temperature to 104° F. or more after the injection of abortin may possibly be indicative of infection, but it will be necessary to carry out a large number of tests in practice before deciding upon the value of the method of diagnosis."

OSTERTAG'S STREPTOCOCCUS IN ABORTION IN MARES.

An extensive epizoötic of abortion among mares was studied in 1899 and 1900 by Ostertag. While he expected to find as its cause Bang's bacillus, he failed entirely in spite of the use of the proper culture medium to encounter this organism, but instead of it, he obtained from the edematous fluid between the uterine mucosa and the fetal membranes, from the heart's blood, the pleural fluid, and the gastric contents of the dead feti a *short, immobile, Gram-negative streptococcus*. The organism was difficult to cultivate; it grew best in *serum bouillon, serum agar,* and the *transudate of dead feti*. The fluid media are clouded by these growing streptococci in two days,

and after two more days a sediment is formed. On *serum agar* the growth is very delicate and scarcely visible to the naked eye. The organism does not grow on *gelatin* or in *milk*, and very poorly in nutrient bouillon without the addition of serum. Transplants cannot be kept up long on any medium; they generally fail after the fifth generation. Ostertag injected cultures of this streptococcus into two pregnant mares. The one which was injected intravenously aborted after twenty days; the other one, inoculated into the vagina, went to full term, but gave birth to a very weak foal.

These streptococci are not pathogenic for either mice, guinea-pigs, or rabbits; they are very slightly resistant to disinfectants. The natural infection is brought about by sexual intercourse.

STREPTOCOCCUS OF VAGINITIS VERRUCOSA OF THE COW.

An infectious vaginitis in cows is relatively prevalent in several European countries. It has been particularly widespread in Eastern Germany. The disease is known under the names of *kolpitis granulosa, infectiosa bovum, vaginitis verrucosa;* "Ansteckender Scheidenkatarrh der Rinder" or "Knötchenseuche" (German). The affection is chronic in character and does not yield easily to treatment. When healthy cows are infected experimentally from the vaginal discharges of sick animals, swelling, redness, and tenderness of the vaginal mucosa appear after two to three days. Afterward the lymph follicles of the mucous membrane swell up and form granules. It is this change which has led to the name granular vaginal catarrh of the cow. Ostertag and Hecker discovered as the cause of the disease a short streptococcus of generally six to nine individual cocci, which are surrounded by a capsule. This streptococcus is found not only in the discharge, but it penetrates deep into the epithelial layers and into the papillæ of the vaginal mucosa. The organism stains best with Loeffler's alkaline methylene blue; it is Gram negative. The streptococcus can be easily cultivated on *glycerin agar, coagulated blood serum,* in *gelatin,* and in *bouillon.* The latter becomes diffusely cloudy. The organism does not liquefy gelatin or coagulated blood serum; it does not coagulate *milk,* nor form hydrogen sulphide, indol, or gas in media containing glucose.

Animals Susceptible.—The ordinary laboratory animals are not susceptible to infection with this organism. Infected vaginal discharges of cows or pure cultures of the streptococcus produce the typical disease in healthy cows, if inoculated into their vaginæ. Sheep, goats, horses, and hogs cannot be infected in this manner. In addition to the specific streptococcus the vaginal discharges of cows suffering from the diseases generally also show staphylococci and colon bacilli. Bulls spreading the disease from sick to healthy cows are generally not made sick, exceptionally, however, they likewise develop a catarrhal discharge from the penis.

Resistance.—The resistance of the organism to disinfectants is of a very low degree, yet its eradication in an infected vagina is difficult because it penetrates deeply into the tissues of the mucosa.

QUESTIONS.

1. What kind of disease is abortus enzoöticus of cattle?
2. To what is it due? Who discovered its microörganisms?
3. Describe the characteristic pathologic lesions of the affection.
4. Describe Bang's abortion bacillus as found in the exudate between the uterine mucosa and the fetal membranes.
5. How is the disease spread under natural conditions?
6. What animals are susceptible to artificial inoculation?
7. Describe the morphology of the bacillus of infectious abortion.
8. Describe the preparation of Stribolt's gelatin-agar-serum mixture.
9. How does the Bang bacillus grow in it?
10. What are the relations of the growth of this organism to oxygen?
11. Discuss the resistance of the bacillus.
12. Discuss the diagnosis of the disease.
13. What is the cause of infectious abortion in mares? Describe the organism.
14. Describe its cultural properties.
15. What is the cause of infectious granular vaginitis in cows?
16. Describe the pathologic lesions in kolpitis granulosa infectiosi bovum.
17. Why is the disease comparatively resistant to treatment?
18. Describe the morphologic and cultural properties of the streptococcus of bovine vaginitis.

CHAPTER XXVIII.

TUBERCULOSIS—DISTRIBUTION AMONG MAN AND ANIMALS—ROUTES OF INFECTION.

THE term tuberculosis is derived from the Latin word *tuberculum*, the diminutive of *tuber*, which means a knob, protuberance, or nodule. It is a very widespread disease among man and some of the domestic animals, particularly cattle and swine, and is characterized anatomically by the formation of poorly vascularized or avascular nodules, which have a tendency to become caseous. The disease in all of its forms is due to a specific organism known as the *tubercle bacillus* of Robert Koch.

Historical Remarks.—The pulmonary form of tuberculosis of man is also termed consumption, or phthisis, *i. e.*, a consuming or wasting away, and has been known to mankind for thousands of years. A vivid description of the disease is found in the works of Hippocrates, but it would hardly have enabled the Father of Medicine to pass a very creditable examination as to etiology and morbid anatomy. Hippocrates[1] distinguished three forms of pulmonary tuberculosis in man, and his description is so highly interesting that a few passages from it will not be out of place. He says: "There are three kinds of pulmonary consumption. The first kind is due to mucus. If the head, filled with mucus, becomes diseased and heated the mucus in the head becomes putrid because it cannot be removed properly. After the mucus has become condensed and putrid, and after the bloodvessels have become overfilled with it, a flow toward the lungs is established, and as soon as the lungs contain this mucus they become corroded because the latter is salty and turbid. The patient now experiences a mild fever, with chills, the chest and the back are painful, he is tortured by a violent cough, and he coughs up large masses of a moist, salty sputum. This is what he experienced in the beginning of the disease, and in its farther course the body wastes, with the exception of the legs, which swell up, also the feet swell, and the nails become contracted. He becomes lean around the shoulders, the larynx becomes filled with a kind of foam, and the breath whistles like the air in a reed. During the disease the patient has great thirst; he becomes very weak. If he is in this condition, as a rule, he perishes miserably from the wasting away within a year.

[1] The quotations are taken from the German translation of *Hippocrates'* works, by Robert Fuchs, Munich, 1897, vol. ii, chap. *x*, p. 494 et seq.

"The second kind of pulmonary consumption is due to overwork, and the third kind to an overfilling of the bone marrow and blood-vessels, with watery mucus and bile."

The treatment recommended by Hippocrates is chiefly dietetic and hygienic—asses', cows', and goats' milk, boiled or raw, honey, etc. He also recommends a good deal of walking in the open air, with care not to take cold. In spite of treatment, Hippocrates says the disease generally takes a fatal course.

It appears that the ancient Hebrews recognized tubercular lesions in cattle, because the Talmud refers to morbid changes found in slaughtered cattle, and it interdicted the use of meat from animals with such ulcerative, evidently tubercular, lesions.

After the writings of Hippocrates little progress was made in the study of consumption for many hundreds of years, but from time to time the belief that it was an infectious disease sprang up, only to be forgotten again.

Silvius and Morgagni were the first to point out the inter-relation between the formation of nodules and their subsequent breaking down and ulceration in the production of pulmonary phthisis. Silvius considered the disease contagious. Bayle and Laennec were the first investigators of tuberculosis who recognized the importance of caseous material in the natural history of the disease. Laennec recognized the identity of tuberculosis and what was called scrofulosis. Virchow established the anatomical diagnosis upon the basis of the poorly vascularized inflammatory nodule, which later on undergoes caseation. Virchow, however, did not believe tuberculosis in man and pearl diseases in cattle to be one and the same disease; on the contrary, he considered pearl disease in cattle a form of lympho-sarcoma (*i. e.*, a form of malignant tumor formation). It is interesting to note here that tuberculosis in cattle at the beginning of the eighteenth century was looked upon as a form of syphilis, due to sodomitic contact with infected persons. Before this time, however, and later, in spite of Virchow's great authority, certain investigators held that pulmonary tuberculosis in man and cattle were one and the same disease (Gurth, Hering, Fuchs), and others even believed that pearl disease of the peritoneum in cattle and human tubercular peritonitis were identical.

The first inoculation experiments with material believed to be tubercular were made by Kerwin (1789), Lepelletier, Goodlad, and Desgallieras. The experiments were undertaken on human beings, but they were, fortunately for them, but unfortunately for science, not successful. Klenke (1843) was the first to report a successful production of tuberculosis by intravenous inoculation of a rabbit with tubercular material, but his work did not attract much attention, and was pronounced inaccurate by some who inoculated apparently indifferent material and produced tuberculosis. Villemin presented his celebrated contribution on the "Cause and Nature of Tuberculosis

and the Inoculation of the same from Man to Rabbits," in December, 1865. From his successful experiments he drew the conclusions that tuberculosis is a specific disease, that it has its origin in an inoculable virus, which can be transferred successfully from man to the rabbit, and that it is a virulent disease, which should be classified with smallpox, scarlet fever, syphilis, and which can be likened to glanders. Villemin's inoculation experiments with tubercular material were successfully repeated by some; others apparently produced tubercular lesions with morbid, but not tubercular, material and with pieces of glass, rubber, wood, etc., so that Villemin's views were soon discredited. The work of Schueller, Tappeiner, Langhans, Cohnheim, Salmonsen, Baumgarten, Klebs, Chaveau, Bollinger, Kitt, Gerlach, and others, undertaken after the publication of Villeman's investigations and continued until the year 1881, gradually demonstrated more and more clearly the infectious nature of tuberculosis. Klebs, Toussaint, and others made attempts to cultivate the unknown living virus of tuberculosis, and Aufrecht and Baumgarten undoubtedly saw tubercle bacilli which they were unable either to stain or to cultivate. It was Robert Koch, however, who finally succeeded in establishing the etiology of tuberculosis, this most important disease of man and many of the domestic animals.

The cultural methods devised by himself, the experiments he undertook, his deductions and conclusions drawn from them, and his first formal communication are even today a monument and model of classical experimental research work in medicine. Some of those who have followed in his footsteps and profited by his pioneer work appear to have forgotten the debt owed him and have venomously attacked him for the view he took as to the intertransmissibility of bovine and human tuberculosis. This question is as yet by no means fully settled, but in the light of researches made during the last decade it certainly appears that Koch's standpoint was too radical.

Distribution in Man.—Tuberculosis in human beings occurs almost over the entire world. It is absent only at very high altitudes. It occurs at all ages, but the greatest number of advanced cases are found in the middle years of life. The United States Census of 1890 showed that with a population of 76,000,000 there died in that one year 111,059 persons from tuberculosis of the lungs, or about one-ninth of the deaths from all causes were due to pulmonary tuberculosis. In Germany the mortality was still larger, reaching 118,706 in a population of 56,000,000. These figures include only deaths from the pulmonary form of tuberculosis, and convey an incorrect idea of the prevalence of this disease among the human race, because pulmonary tuberculosis is often very chronic, and exists for a long time before it leads to death, and tubercular infections are by no means always fatal, as is frequently believed among the laity. On the contrary, they often heal spontaneously, and death results from different causes in individuals with healed

tuberculosis or affected with its latent form which may lead to recovery, or to a more actue, eventually fatal outbreak. The most careful postmortem examinations on human material show that probably not less than 70 to 90 per cent. of all persons, no matter from what disease they may die, have at some time had a tubercular infection. Pulmonary tuberculosis has often been found absent among uncivilized tribes living in a state of nature, but contact with civilized races soon causes it to appear among them and often to become frightfully prevalent, as among the Indians and negroes in the United States. Another good example of the same tendency is found among the inhabitants of the Philippine Islands, who before the advent of the Spaniards were apparently free from tuberculosis. Now, tuberculosis is frightfully prevalent among those tribes which have come into contact with the civilized races for several hundred years. One encouraging feature in regard to the prevalence of tuberculosis lies in the fact that the mortality and evidently also the number of persons affected has diminished since its cause and manner of spreading has become known and made precautions and prophylaxis possible. In Germany the death rate per year per 1000 inhabitants has fallen from 3.1 to 1.9 from 1890 to 1903; and in the United States from 1890 to 1900, from 2.544 to 1.095 per year per 1000 inhabitants.

Tuberculosis in Animals.—Tuberculosis among animals, in the wild state, both mammalians and birds, is practically unknown; but it is quite prevalent among domestic animals and among wild animals confined in zoölogical gardens, menageries, etc. Monkeys, which in nature are never found to be infected with tuberculosis, generally die from it in captivity; guinea-pigs and rabbits, which are the most susceptible common laboratory animals, and very frequently used in inoculation experiments, are rarely affected spontaneously even in captivity. Antelopes, giraffes, zebras, wild animals of the canine and feline tribes, often contract the disease in confinement. Wild birds in aviaries often contract the avian type of the disease, but with the exception of parrots, rarely the mammalian type.

Tuberculosis has also been observed in goats, sheep, dogs and cats and quite frequently among domestic fowl.

Cattle.—Tuberculosis among cattle running wild on extensive steppes and prairies is rare, but when the animals are kept in barns, crowded and subjected to stable feeding, it becomes very common. It reaches its highest percentage among *milch cows,* which are often kept under the most unnatural and unhygienic conditions. There are cases on record where more than one-half and up to 80 per cent. of a herd of milch cows have been found infected.

Hogs.—The disease has become widespread among hogs since it has become customary to feed them on skimmed milk which has been returned from the creamery. The milk from a few cows affected with obvious or latent tuberculosis, when mixed with a large amount of milk from unobjectionable sources, may infect the entire quantity,

and by its return in small lots to a number of farms may spread tuberculosis among hogs to which the skimmed milk is fed raw. Mohler and Washburn have recently dealt with this subject in a paper presented at the 44th annual meeting of the American Veterinary Association in Kansas City. According to their figures, in three cities in one of the leading dairy States, from 3.1 to 6.4 per cent. of all hogs slaughtered were found affected with tuberculosis. Hogs also frequently contract tuberculosis by being fed on the same premises with tuberculous cattle, or by being allowed to feed on offal from slaughter houses or to devour the carcasses of cattle dead from tuberculosis. In a case cited by Mohler and Washburn of a hog raiser who fed 40 hogs on the carcass of a cow dead from tuberculosis, 31 of the 40 had to be condemned for tuberculosis when they came to be slaughtered. Hogs also easily acquire tuberculosis when they are taken care of by tubercular persons who cough and spread their sputum promiscuously in the pigpens.

Modes of Infection and Transmission.—It was formerly believed that tuberculosis was most frequently inherited from parents by the offspring. This view has now been entirely abandoned and the inhalation and the ingestion of tubercle bacilli are recognized as the most frequent modes of infection.

Inhalation.—In man, tuberculosis is most frequently, and in adults almost exclusively, contracted by the inhalation of tubercular material from previously infected individuals. The sputum coughed up at frequent intervals by patients suffering from pulmonary tuberculosis contains many millions of tubercle bacilli. These may be inhaled directly with fine moist particles floating in the air for some time or the sputum may be dried out and pulverized and the dust particles inhaled. It was first shown by Cornet that the dust accumulating in rooms, wards, barracks, prisons, etc., where consumptives had been allowed to spit on the floor, contained live, virulent tubercle bacilli. Ever since that time efforts have been made throughout the civilized world to prevent consumptives from spreading their tubercular sputum promiscuously and to enforce the immediate destruction of the infected sputum by collecting it in proper receptacles containing a proper antiseptic. This simple measure has probably contributed more to the reduction of pulmonary tuberculosis than any other single measure. There are two theories as to the development of tuberculosis from inhaled tubercle bacilli. According to the older, more generally accepted view, the tubercular process develops directly in the pulmonary alveoli. According to a more recent view, the tubercle bacilli are first absorbed through the bronchial mucosa, taken up by the lymphatics, and carried to the bronchial lymph glands, where they multiply and from which point they later invade the parenchyma of the lungs.

Inhalation tuberculosis is also very common among cattle. In dairies a single cow coughing up tubercular material often spreads

it to other cows. Cows infected with pulmonary tuberculosis also swallow a considerable quantity of their sputum and disseminate the bacilli through their feces, which may contaminate the fodder of other cows.

Ingestion.—This is the most common mode of infection in hogs. Its process through skimmed milk from tubercular cows, feeding after infected cattle, and devouring tubercular offal or cadavers has already been referred to. Tuberculosis is also contracted through the intestinal tract by calves feeding on cows with either more or less generalized tuberculosis or with udder tuberculosis.

Von Behring has advanced a theory that tuberculosis in man is almost always primarily an intestinal infection in which the tubercle bacilli are taken into the body in early childhood with milk from tubercular cows. The bacilli then remain latent in the abdominal cavity, particularly in the mesenteric glands. Later they enter the lymphatics or the blood circulation and find the lungs the most favorable place for their development and the establishment of an extensive tubercular process. Certain statistics have been produced in opposition to von Behring's view. They show that among a large number of pulmonary consumptives, only a very small percentage was fed on cow's milk as infants, while the great majority was breast-fed and had never even received very much cow's milk. A powerful argument indicating that pulmonary tuberculosis is developed independent of feeding with cow's milk is the following, which the author has not found mentioned elsewhere. Most monkeys and apes kept in zoölogical gardens die from pulmonary tuberculosis. Most of these animals have been born in tropical or subtropical countries. They have, of course, been breast-fed by mothers never sick of tuberculosis in the wild state, and have probably, throughout their lives, never had a drop of cow's milk. Yet in captivity these animals almost invariably die of pulmonary tuberculosis which they contract by inhaling moist floating particles of pulverized dust of dried human tubercular sputum. Another very strong argument against the ingestion theory of von Behring is furnished by the observation that certain nations, among which pulmonary tuberculosis is very prevalent, do not consume cow's milk nor keep cattle, tubercular or otherwise, as domestic animals. The inhabitants of Japan and the Philippine Islands which, for example, are such countries, keep water buffaloes, or carabaos, as beasts of burden, but not as meat- or milk-producing animals. There is no tuberculosis among these carabaos, and as the cattle of the Western nations are not kept among the Eastern nations named, they cannot have played any role in the great prevalence of tuberculosis among the Japanese and the Filipinos, nor can the disease of the lungs be traced back to an early infection with bovine tubercle bacilli.

Wounds.—Wounds of the external surface play a relatively unimportant part in producing tubercular infections in man and animals.

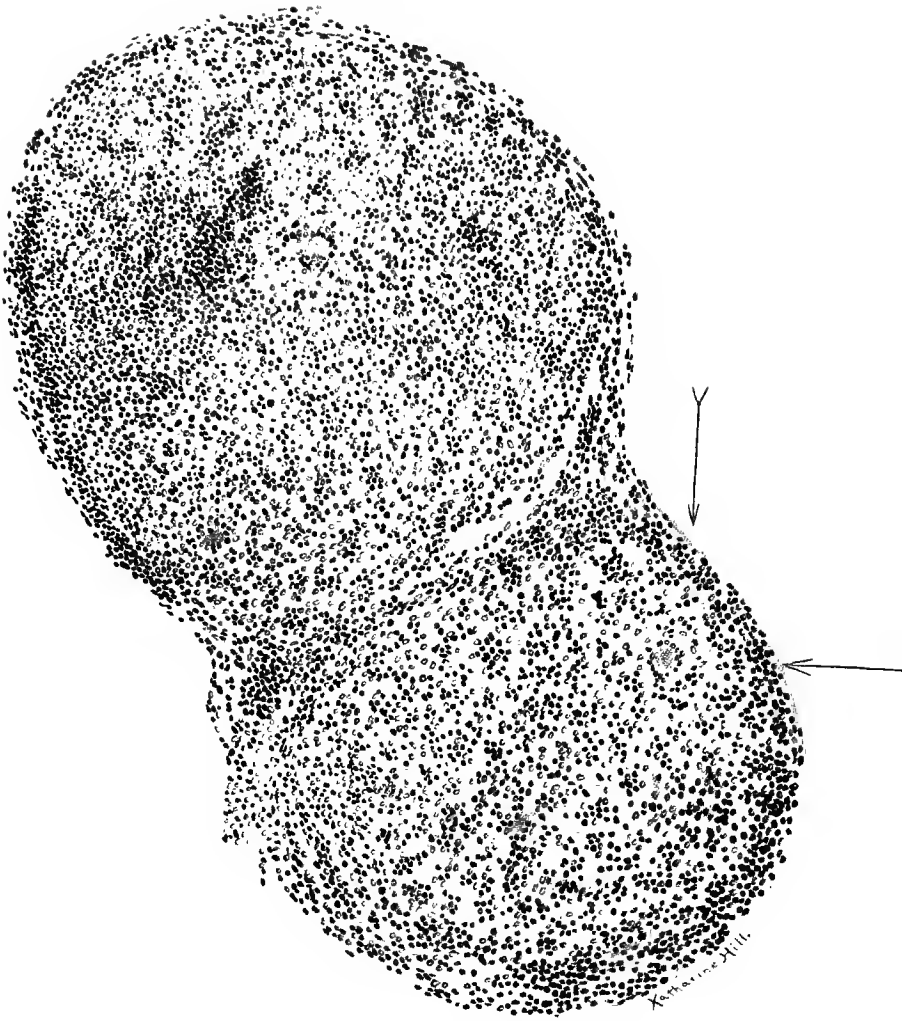
Sexual Intercourse.—Sexual intercourse in cattle, one of the participants having been affected with tuberculosis of the sexual organs, has led to an infection of the healthy animal, but such observations have been very rare. There is not the slightest proof that tuberculosis is ever spread through spermatozoa from the male to the developing ovum. Tuberculosis, however, is occasionally spread from a tuberculous mother to the offspring *through the placenta.* These cases are rare in human beings, but more common in cattle.

The question of intertransmissibility of human and bovine and mammalian and avain tuberculosis will be discussed in another chapter.

QUESTIONS.

1. What is the derivation of the word tuberculosis? What is the cause of the disease in its various forms?
2. Who furnished the earliest description of the symptomatology, etc., of pulmonary tuberculosis?
3. What is the derivation of the term phthisis?
4. Who made the first inoculation experiments with what was considered tubercular material on man? What was the outcome of these experiments?
5. Describe the experiments of Villemin and their results.
6. Discuss the prevalence of tuberculosis among human beings.
7. To what percentage are tubercular lesions found in man according to postmortem statistics?
8. Discuss the prevalence of tuberculosis among uncivilized nations living in a wild state. What if such nations come into contact with civilized people among whom tuberculosis is of common occurrence?
9. Is tuberculosis among human beings on the increase or decrease?
10. What is probably the most important single method of combating the spread of tuberculosis among human beings?
11. What animals suffer from tuberculosis?
12. How is tuberculosis most commonly spread among human beings?
13. How most commonly among cattle?
14. How most commonly among hogs?
15. Discuss the various routes of infection by which tuberculosis invades the body of susceptible beings.
16. What are the two different views as to the most common mode of development of pulmonary tuberculosis?
17. Why is it improbable that pulmonary tuberculosis in man is generally due to the ingestion in infancy and childhood of bovine tubercle bacilli?

PLATE VIII



Section from the Tubercular Penis of a Bull.

——> Indicates position of a giant cell. Zeiss objective 3 mm.; compens. ocular No. 6.

CHAPTER XXIX.

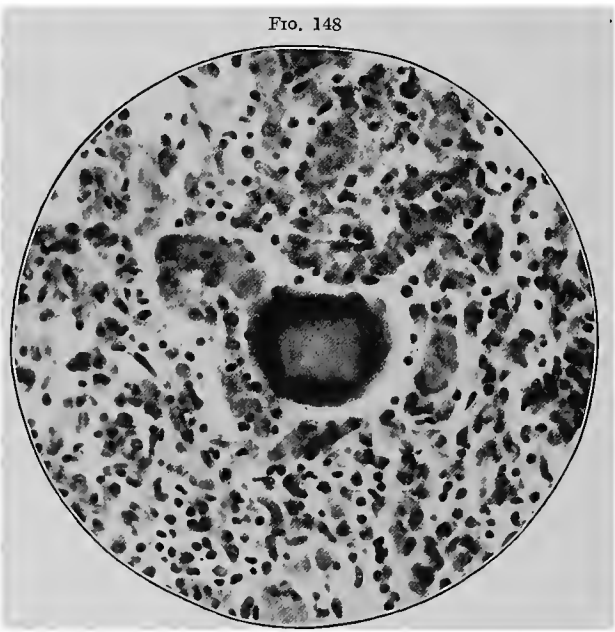
TUBERCULOSIS (CONTINUED)—HISTOPATHOLOGY AND MORBID ANATOMY IN MAN AND ANIMALS.

The Tubercles.—The characteristic anatomical lesion of tuberculosis is the small, avascular, nodular mass of granulation tissue, called the tubercle, which has a very pronounced tendency soon to undergo retrograde, degenerative changes. The formation of the tubercle can best be studied experimentally, as has been done by a number of investigators, including Cohnheim, Baumgarten, and others. It is accomplished by injecting finely divided tubercular material or tubercle bacilli from a pure culture which has been rubbed up and diluted with physiologic salt solution into the anterior chamber of the eye of a rabbit. Small, grayish-white, first perfectly translucent nodules develop in the eye, within twelve to fifteen days. Their size when first seen is perhaps not larger than a pinhead, becoming larger during the next few days and at the same time less translucent; in fact, their centres become opaque. Neighboring nodules become confluent and in this manner larger grayish-white or yellowish-opaque nodules are formed. When infected tissues are obtained from a series of animals from day to day and properly fixed, embedded, sectioned, and stained, for microscopic study, they show first a slow multiplication of the injected tubercle bacilli. These, or rather their metabolic products or endotoxins, cause some of the ordinary fixed connective-tissue cells and some lymphatic endothelial cells to proliferate much more rapidly than they do under normal conditions. This increased rate of cell multiplication or proliferation is indicated by the presence of a considerable number of *karyokinetic figures.*

The new cells formed under the stimulus of the infection with tubercle bacilli become larger than the ordinary connective-tissue cells in normal adult connective tissue. They possess a vesicular nucleus and a rather large, round, or polygonal body of protoplasm. They are, in other words, the cells generally known in histopathology as *epithelioid cells.* A number of investigators claim that the first effect of the presence and multiplication of tubercle bacilli in connective tissue is the cell proliferation just described. Others hold that the very first effect is a coagulation necrosis of the connective-tissue cell in the closest proximity to the multiplying tubercle bacilli, that this necrosis leads to a moderate transudate with the migration of leukocytes, and that the proliferation of the fixed connective-tissue cells begins only after this has occurred. It appears that the preponderance

of evidence is in favor of the former view that the stimulus to proliferation and the latter itself *precede the coagulation necrosis.* In any event, if necrosis occurs first, it is very insignificant, and the proliferation with the appearance of karyokinetic figures is very obvious and impressive in the first stage of the formation of the tubercle. In this first stage the tubercle bacilli are found partly between the cells and partly inside of the protoplasm of epithelioid cells. Whenever bacilli have gained entrance into the cell protoplasm, they evidently cause a profound disturbance of the cell metabolism. The bacilli-infected cell grows and becomes larger by the absorption of nutritive material and its nucleus divides, but not the cell protoplasm itself.

FIG. 148



Section through tubercular tissue of pleura of a horse. (Author's preparation.)

This process continues, and very large cells with many nuclei are formed. The latter are frequently distributed around the periphery. The bacilli infecting such a *large multinuclear giant cell* are generally found in the protoplasm at some distance from the nuclei. The protoplasm of the giant cell early shows evidences of necrobiosis or coagulation necrosis or granular degeneration. While such giant cells are forming the epithelioid cells increase in number. Among them, and particularly around them at some distance from the point where the bacilli are most numerous, small round cells with round, granular, deeply staining nuclei and small protoplasmic bodies appear. These cells are proliferated young connective-tissue cells, which have,

however, not attained the larger size and the other characteristics of the epithelioid cells. These are called *lymphoid cells.* While all these newly formed cells appear and accumulate, they push aside to a great extent the fibrous matrix of the connective tissue in which they have developed. In consequence of this the young cellular tubercle shows a rather scanty fibrous reticulum. If, however, the cell proliferation has remained within moderate limits, when few tubercle bacilli have been present and when they are, perhaps, of a low type of virulency, or when the process has come to a standstill, or when, in consequence of natural or artificial protective influences, a process of healing is setting in, and when some of the newly formed inflammatory cells have disappeared in consequence of softening, liquefaction and absorption, *the fibrous reticulum* may become quite abundant, particularly at the periphery. During the formation of the tubercular inflammatory granulation tissue, in other words, the tubercle, there is a complete *lack of the formation of bloodvessels* in the newly formed tissue. In this respect the tubercle differs greatly from ordinary inflammatory granulation tissue in which vascular buds and new vessels are formed. The more virulent the infecting bacilli the more rapid their multiplication, the sooner the retrograde degenerative processes become manifest and the more extensive they are likely to be. The early degeneration of the tubercle is probably not only due directly to the metabolic products of the tubercle bacillus, but likewise to the fact that new vessels are not formed and that preëxisting ones are obliterated in the mass of the newly formed cells of the tubercle. For this reason the tubercle from the beginning suffers from a lack of proper nutrition.

From the preceeding description it follows that a typical young tubercle, presented to the unaided eye as a small, grayish-white, perfectly translucent nodule generally contains three kinds of cells, namely, *multinuclear giant cells, epithelioid cells,* and *lymphoid cells,* all of which are derived and descended from fixed connective-tissue cells. Such young tubercles occasionally also show some *polynuclear leukocytes* which have wandered from the bloodvessels into the newly formed inflammatory granulation tissue. Sometimes when the tubercular infection is very mild, moderate, and slow in its action upon the infected connective tissue there occurs simply an accumulation of and an infiltration with small lymphoid cells between which an epithelioid cell may be seen here and there, particularly in thin sections. Such tubercles, composed almost exclusively of lymphoid cells, with only a very few epithelioid cells, but without any giant cells whatsoever, are called *lymphoid tubercles.* Unless there is other evidence at hand they cannot be distinguished from an ordinary subacute inflammatory small round-cell infiltration or focus.

Caseation.—The degeneration of the tubercle is known as its caseation, because there is formed a rather dry, but soft, friable material of the consistency and appearance and other physical properties

of soft cheese. Caseation was looked upon by Weigert and others as a particular form of *coagulation necrosis*. It begins, in fact, in the tubercle with the appearance of the giant cells. Their protoplasm early shows evidences of a coagulation necrosis, and not infrequently in giant cells nuclear fragments are found in a necrotic coarsely granular protoplasm. When the process of caseation progresses in the giant and other cells of the tubercle its centre becomes completely necrotic. The cells and their nuclei break up into a *granular detritus* which take both the eosin stain and to some extent, but diffusely or irregularly, the nuclear stain. During the formation of the caseous material in the centre of the tubercle there is a certain amount of transudation from the neighborhood of the tubercle and the necrotic material becomes infiltrated with a coagulable substance which, while not the true fibrin, is evidently much like it, and hence is called a *fibrinoid substance*. The latter is to a large extent responsible for the particular rather dry character and consistency of the caseous material. Its characteristics are also partly due to the lack of vessels and depending upon it the lack of fluids in the tubercle. The tubercle which has undergone caseation in the centre now appears somewhat yellowish; it has lost its transparency and has become opaque and cloudy. At this stage of retrogressive changes, giant, epithelioid, and lymphoid cells are seen peripherally to the caseous centre and between them appear polynuclear leukocytes which even wander into the caseous centre itself, where some of them may likewise become necrotic or may remain unchanged and active to perform their phagocytic function. Still later the caseous material becomes softened and more creamy. This softening is probably brought about by digestive proteolytic ferments, which are transported to the caseous centre by the transudate and by the wandering leukocytes. If by this time the activity of the bacilli has become lessened, the development of *fibrous connective tissue* at the periphery becomes more abundant and a *fibrocaseous tubercle* develops. If the caseous material becomes completely liquefied and absorbed the inflammatory cells disappear more and more and the originally cellular and partly caseous tubercle becomes completely replaced by fibrous connective tissue, and in place of the original cellular tubercle there is now a *fibrous nodule*. As the tubercle disappears its elements may be replaced by a deposit of *lime salts*, and this process is termed *calcareous degeneration of the tubercle*.

The inflammatory cells of the tubercle in the degenerative processes generally become necrotic. According to Hektoen's observations, sometimes in healing tuberculosis, young not yet necrotic giant cells may break up into mononuclear cells, and these as fibroblasts form connective-tissue fibers.

The change from a tubercle into a fibrous nodule generally occurs in healing tuberculosis. Even while this occurs new tubercles may be formed in the neighborhood and the process spread. Even calcified

tubercles or fibrous nodules may still contain live virulent tubercle bacilli. These finally disappear entirely or they remain latent for a long time, and later lead to a fresh outbreak of the tubercular process. Fibrosis, caseation, and calcification may also continue more or less simultaneously in numerous tubercles in a larger area and lead to the formation of large masses of fibrous connective tissue, caseous or calcereous material.

Cryptogenetic Tubercular Infection.—The tubercle bacillus, as already pointed out, generally enters the body of man and the susceptible lower animals through the respiratory passages or through the gastro-intestinal tract. It is in these parts and their lymph glands that the tubercular lesions generally first make their appearance. The bacillus, however, may enter the system through its usual portals of entrance and may not produce morbid changes in those places, but be carried away by the lymph or blood current to produce its first lesions at places which have no direct open communication with the outside world. Such places are, for instance, the bones, the periosteum, and the synovial membranes of the joints, the brain, the testicles, etc. This is known as a cryptogenetic infection, which means an infection of secret or hidden origin. *Tubercular infections, however, which do not first involve the regional lymph glands near their portal of entrance are, on the whole, very rare.*

Fungous Tubercular Granulations.—The tubercle bacillus may become localized from the beginning, not merely at a single point, but over a larger area of a certain structure. It may also rapidly spread locally and lead to the formation of numerous tubercles between which, in consequence of the inflammatory irritation, a great deal of ordinary inflammatory granulation tissue develops in which the tubercles lie embedded. These soft spongy masses which develop are called fungous tubercular granulations. They are seen particularly in the synovial membranes of the joints and the bursæ.

Solitary Tubercles.—Sometimes masses of tubercular granulation tissue form a single round or oval mass of the size of a hazel nut or walnut. Tubercles of this kind are called solitary tubercles. They are not infrequently seen in the brain or its membranes of man and cattle. These round masses, of course, do not represent one tubercle, but are made up of numerous miliary and submiliary tubercles.

Fibrosis and Hyperplastic Tuberculosis.—As has already been stated the tubercle may in healing undergo a fibrosis and become changed into a fibrous nodule. When this process continues in a larger number of tubercles over a larger area it leads to the formation of a considerable amount of fibrous connective tissue. It also occurs, particularly in the intestines of man, that a tubercular process is comparatively mild from the beginning and does not give rise to many cellular tubercles, with subsequent caseation, but rather leads to the formation of a very large amount of fibrous connective tissue. This form of tuberculosis is known as a hyperplastic tuberculosis.

Caseous Infiltration.—When many tubercles in the same area undergo caseation, and when these caseous masses become more or less confluent and form one larger territory, the process is termed caseous infiltration of a part or organ. This form of caseous infiltration can best be seen in the lungs of cattle before cavity formation has occurred and in the lymph glands. The tissues of these structures become infiltrated by a rather dry, elastic, cheesy material, generally grayish white or light grayish in man, and more decidedly yellowish (like the boiled yolk of an egg) in cattle. In the latter these caseous infiltrations may lead to the formation of masses of several pounds' weight. When they have attained such a large size they are frequently riddled with areas where the caseous material has been softened and even completely liquefied.

Cold Abscess and Cavity Formation.—When the caseation and liquefaction of neighboring tubercles lead to the formation of a larger abscess filled with more or less liquefied material which may be discharged through an open ulcer or a fistulous tract the process is known as a tubercular or a cold abscess. Such an abscess is generally surrounded by a wall, on the inner free surface of which may be seen miliary and submiliary tubercles. When these abscesses have been formed in the lungs and have discharged part of their fluid contents they are known as *pulmonary cavities*, or *caverns*. They generally break by ulceration or the formation of a fistulous tract into a bronchus, and discharge their contents by coughing. Such cavities, as an invariable rule, sooner or later become infected from the outside by other microörganisms (staphylococci, streptococci, Bacillus pyocyaneus, etc.). This process is known as a *mixed tubercular infection*. Tubercular lesions of the intestines, after they have ulcerated, generally become infected with the colon bacillus. In tuberculosis, abscess formation occurs not only in soft tissues, but it may also take place in the bones. Here the tubercular abscess or cavity generally contains bone fragments or bone sand, and also occasionally complete sequestra floating in the softened tubercular material; sometimes the area where the bone has been broken down and carried away is filled with a fungous tubercular granulation tissue.

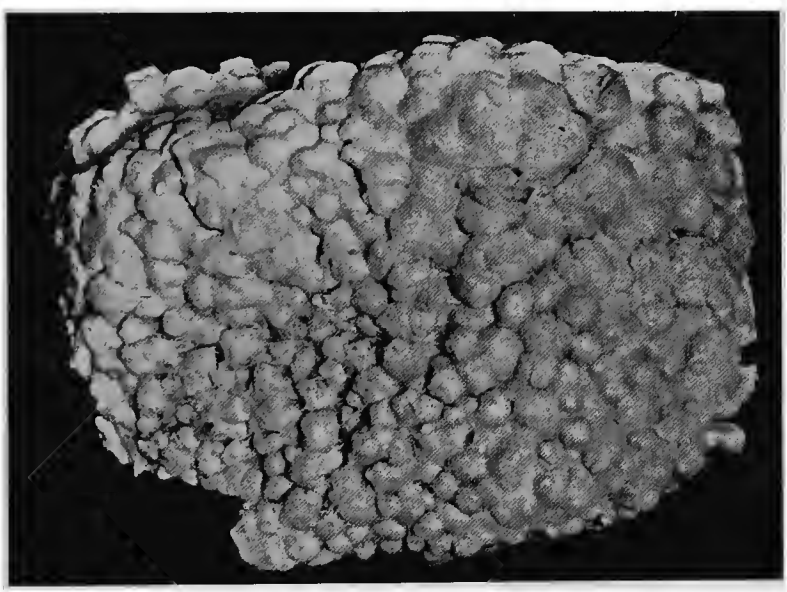
Tubercular Ulcers.—Tubercular ulcers are frequently found in the intestines, the larynx, trachea, and bronchi as a result of tubercles or confluent tubercles which have first undergone caseation in the centre, the necrotic process having broken through the outer surface and having led to the formation of open sores. The latter are generally circular or oval, with a ragged, irregular base, grayish-red or yellowish, mottled, often studded with tubercles which surround them at the margin.

Miliary Tuberculosis.—Tubercle bacilli may spread over a larger area, for instance in the pleura, the peritoneum, the liver, or the spleen, in such a manner as to form numerous small tubercles, which

can be recognized individually as nodules of a somewhat larger or smaller size than a millet seed. This form is called a miliary tuberculosis. It is generally due to a simultaneous or gradual distribution of tubercle bacilli by the bloodvessels or the lymphatics of the affected area (hematogenous or lymphogenous infection of an area or organ).

General Acute Miliary Tuberculosis.—It sometimes occurs that a mass of caseous material breaks into a large lymph channel (thoracic duct) or into a larger bloodvessel, generally a vein, and that tubercle bacilli are disseminated in this manner over the entire body. Miliary tubercles are then formed in almost all of the internal organs of the body and a general acute miliary tuberculosis develops. This is an absolutely and rapidly fatal condition which in man may first be mistaken for an attack of typhoid fever.

FIG. 149



Tuberculosis of the pleura in cattle. (Pearl disease.)

Pearl Disease.—The primarily small miliary tubercles usually grow larger, fuse, and in this manner form larger nodules, ranging in size from a pea to a hazel nut. They either become caseous, fibrous, or calcareous, and the entire pleura, particularly in cattle, may be found studded with them. This picture of chronic miliary tuberculosis in cattle has led to the designation "pearl disease."

Tuberculosis in Man.—Almost every organ or part of the human body may be the seat of a primary tubercular infection. The most common form of tuberculosis in man is the pulmonary. Consumption,

or phthisis, generally first appears in the apices of the lungs. The upper respiratory passages are less frequently affected primarily, but primary tuberculosis of the larynx is not rare. Primary tuberculosis of the nose is comparatively rare. The pleura is often involved in phthisis, and it may be involved primarily. The lymphatic tissue, including the tonsils, forms a favorite soil for the development of tuberculosis. Children frequently suffer from a slow grade of tuberculosis of the lymphatic system. In this form of the disease tubercle bacilli frequently cannot be found, or in small numbers only, and the condition was formerly generally known as *scrofula*, or *scrofulosis*. Of the vascular system, the heart muscle itself is rarely the seat of tubercular lesions; the pericardium is involved more frequently. The vessels generally become involved by the extension of a tubercle into the vessel wall, but sometimes submiliary and miliary tubercles may be developed on the intima from bacilli which have been transported by the blood current. The lymphatics, except the thoracic duct, are rarely the seat of tubercles. Of the gastro-intestinal tract the mouth and pharynx are occasionally the seat of the disease, the esophagus and stomach almost never, but the intestines very frequently. The salivary glands and pancreas are rarely the seat of tubercular lesions, the liver and the spleen very frequently, particularly the former. Both the male and female genito-urinary organs are frequently tubercular, particularly the kidneys, testicles, and Fallopian tubes. The adrenals are occasionally affected by tuberculosis, and then a bronze discoloration of the skin occurs. This condition is known as *Addison's* or *bronze disease*. Bones and joints are frequently affected, muscles, fascia, and tendons rarely, except by secondary extension of advanced tubercular processes in other structures. When tuberculosis of the skin occurs as a very chronic, slow, ulcerating process on the hands or face it is known as *lupus*. There is also a rare, warty, granulomatous form of skin tuberculosis called *tuberculosis verrucosa cutis*. The so-called postmortem tubercles of the skin of physicians, veterinarians, butchers, etc., are fairly common.

Tuberculosis in Cattle.—The organs most frequently affected in tuberculosis of cattle are, as in man, the lungs. In the early stages the affection assumes the character of a miliary tuberculosis of one or more lobes. The small grayish-white nodules are found distributed in a generally congested pulmonary tissue. In the later stages larger nodules are seen which, when incised, discharge a dry, caseous, or a more liquid material. Caseation by confluence and agglomeration of numerous large and small tubercles in the latter stages, sometimes involves considerable portions of the lungs, and the process is then known as a caseous infiltration. Cavity formation in the lungs occurs in advanced cases in cattle as in man, while the formation of numerous hard fibrous, caseous, or calcareous nodules in the pleura leads to that picture of tuberculosis in cattle called pearl disease. Tubercular affections of the abdominal organs and the gastro-

intestinal tract is next in frequency. The mouth and tongue rarely present tubercular ulcerations, while the intestines are very frequently affected. The liver and the spleen are often involved, likewise the organs of the genito-urinary tract, particularly the kidneys, testicles, uterus, etc. While tuberculosis of the mammary gland is rare in man, it is common in cattle. Tuberculosis of the udder generally begins as a non-painful, not hot consolidation of the tissues of one or both posterior ventricles of the udder. The area of consolidation spreads slowly, involving the neighboring ventricles and finally forming large (up to the size of a child's head), very hard, uneven, nodular masses which push aside the non-infected parts of the udder and bring about pressure atrophy in them. The tubercular process may also begin from a number of individual nodules, which are at first distinct but later become confluent and fuse into each other. The regional lymph glands become enlarged and hardened. Sometimes the lymph glands above are enlarged and a tubercular focus cannot be detected in the udder, but it is, as a rule, present, though so small that it does not become palpable. The lymph glands in cattle are frequently infected in all forms of tuberculosis. Tuberculosis of the bone is generally not primary but secondary in cattle. Tuberculosis of the central nervous system occurs in the form of *solitary tubercles* and also as a *diffuse cerebrospinal tubercular meningitis. Acute miliary tuberculosis* occurs and generally kills the animal in a very short time.

Nocard and Leclainche give the following figures as to the frequency of the organs involved:

Organs found affected in tubercular male cattle—

Lungs in 70 out of 100 cases.
Visceral pleura in 55 out of 100 cases.
Peritoneum in 48 out of 100 cases.
Costal pleura in 7 out of 100 cases.
Liver in 28 out of 100 cases.
Spleen in 19 out of 100 cases.
Trachea in 3 out of 100 cases.
Intestines in 1 out of 100 cases.
Heart in 0.9 out of 100 cases.
Kidneys in 0.7 out of 100 cases.
Bone in 0.2 out of 100 cases.
Larynx in 0.15 out of 100 cases.
Brain in 0.04 out of 100 cases.
Cord in 0.03 out of 100 cases.
Tongue in 0.01 out of 100 cases.

In generalized tuberculosis the organs affected were found to be—

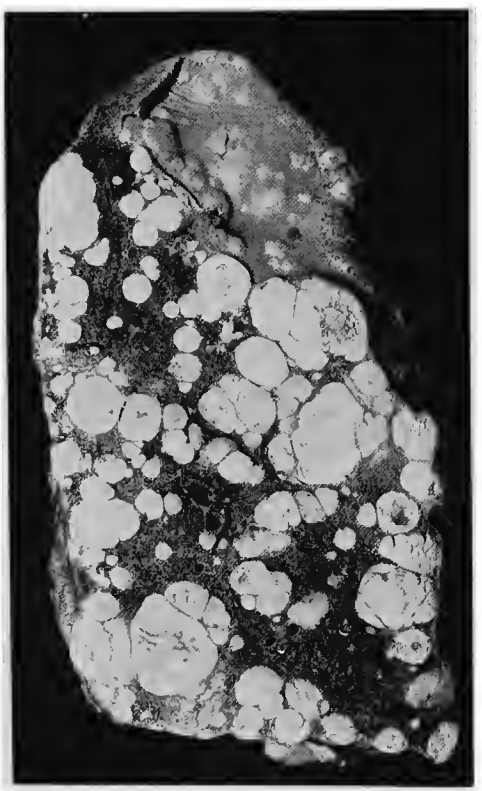
Lungs in 100 out of 100 cases.
Liver in 85 out of 100 cases.
Intestines in 75 out of 100 cases.
Serous membranes in 57.4 out of 100 cases.

Kidneys in 52.2 out of 100 cases.
Muscles in 42.3 out of 100 cases.
Spleen in 18.5 out of 100 cases.
Bone in 8.8 out of 100 cases.

In cows the female genital organs were found affected in general tuberculosis—

Uterus in 65 out of 100 cases.
Udder in 15 to 25 out of 100 cases.
Ovaries in 5 out of 100 cases.

FIG. 150

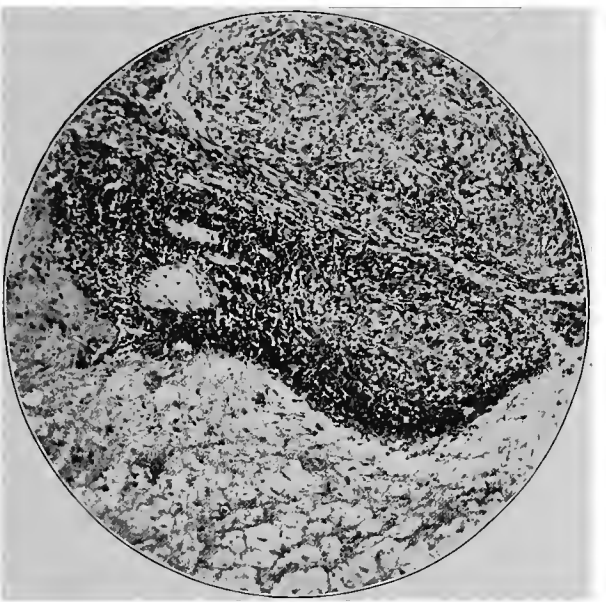


Tuberculosis of the lung of a hog.

Tuberculosis of Hogs.—Since hogs generally contract tuberculosis by the ingestion of food containing tubercular material the lesions are most frequently found in the gastro-intestinal tract and its lymph glands. The tubercular affection of the lymph nodes in hogs is also spoken of as *scrofulosis*. The glands involved are generally the submaxillary, pharyngeal, cervical, mesenteric, sublumbar, and others.

The agglomeration of tubercular glands form irregular and nodular hard, not painful masses up to the size of a fist. Later they become softened, fluctuation followed by ulceration appears, and the fistulous tracts formed discharge a purulent, caseous secretion. The intestines show infiltrations, nodule formation, and ulceration. Tuberculosis of the liver and spleen is common in hogs; the bones and the muscles become secondarily involved. The lungs, the pleura, and the peritoneum generally show the form of a *miliary tuberculosis.* Tuberculosis of the *central nervous system* occurs, but it is rare. *Acute miliary tuberculosis* also occurs in the hog, and is rapidly fatal.

FIG. 151

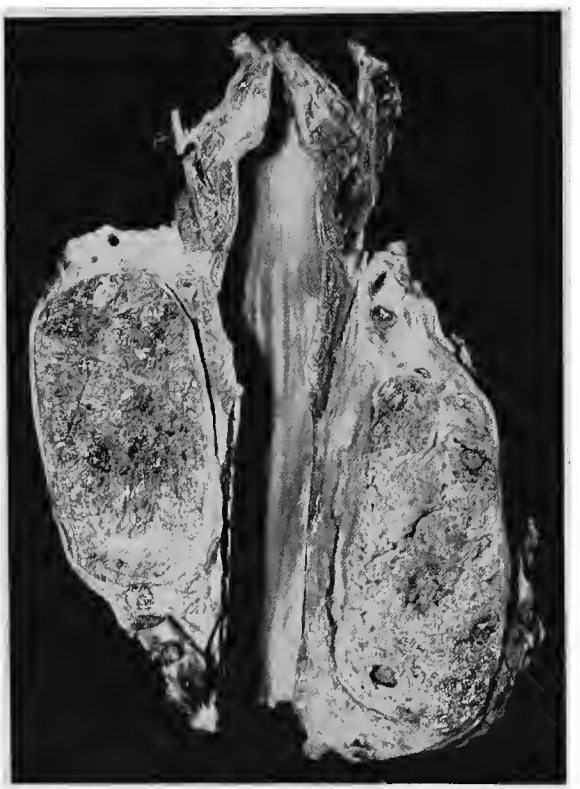


Transverse section through the spinal cord of a hog suffering from tubercular cerebrospinal meningitis. (Preparation by Dr. L. E. Day.)

Histology.—Stewart and Kinsley, who have studied the histology of the tubercle in hogs, found that there are two general types. One type is represented by the cellular necrotic and calcified necrotic tubercle. "This group of tubercular lesions is probably the result of the activity of quite virulent tubercle bacilli. The second type of lesions are those described as fibrous tubercles, which may be preceded by cellular tubercles and usually contain calcareous foci in the later stages; these lesions are no doubt the result of infection with slightly virulent tubercle bacilli. In the examination of 770 sections no giant cells were seen, and it is, therefore, believed that the tissue reaction in hogs against infection with the tubercle bacillus does not favor the production of giant cells."

While this statement is undoubtedly true, giant cells are seen in some tubercular lesions in the hog. The author is indebted to Dr. Enos L. Day for sections of a case of tuberculosis of the skin in a hog. These sections show a number of giant cells; they are, however, not very numerous. Giant cells are also occasionally found in other tubercular lesions of swine.

FIG. 152



Tuberculosis of the bronchial glands of a hog.

Tuberculosis in Other Mammals.—Tuberculosis in goats and sheep is very rare. Tuberculosis in dogs and cats occurs not infrequently when these animals are living with tubercular persons and when they inhale pulverized sputum or ingest food contaminated with it. The most common form of tuberculosis in dogs or cats is that of the lungs or of the intestines.

Tuberculosis in the horse is not very common, though the animal is quite susceptible to artificial inoculation with pure cultures of tubercle bacilli or with tubercular material; but it appears that the

more healthy outdoor life with exercise which this animal generally leads makes it much less susceptible to the natural infection than man, cattle, or swine. When tuberculosis is encountered in the horse it is generally found in young animals in which the mesenteric and other abdominal glands are the favorite seat of infection. These glands are found enlarged and form agglomerated nodular tumor masses; the mesentery and omentum are thickened and the intestinal mucous membrane often shows tubercular ulcers. The tubercular masses may surround large veins, compress and discharge into them tubercular material, bringing about a general acute miliary tuberculosis. In abdominal tuberculosis in the horse the spleen is frequently found involved, the liver rarely. The former may assume a large size and may weigh twenty to twenty-five pounds. Primary pulmonary tuberculosis in the horse generally is of the miliary type; the large tubercular nodules and masses seen in cattle rarely occur; the peribronchial lymph nodes are found enlarged and studded with yellowish nodules, while the respiratory mucosa is sometimes ulcerated. When the serous membranes, the pleura, and the peritoneum are the seat of tubercular lesions in the horse, they present a picture similar to pearl disease in cattle.

Tuberculosis in Food-producing Animals in the United States.—Melvin, in a paper on the *Economic Importance of Tuberculosis of Food-producing Animals*, read before the Sixth International Congress on Tuberculosis, held in Washington in 1908, reported that the following number of animals were found tubercular among a total of 53,973,337 head slaughtered in the United States, under federal inspection, during the fiscal year 1907-08:

Tubercular cattle	68,395
Tubercular calves	524
Tubercular hogs	719,300
Tubercular sheep	40
Tubercular goats	1

Loss for condemned cattle, $710,677; hogs, $1,401,723. The same author estimates the loss due to tuberculosis among meat- and milk-producing animals in the United States at $14,000,000 annually.

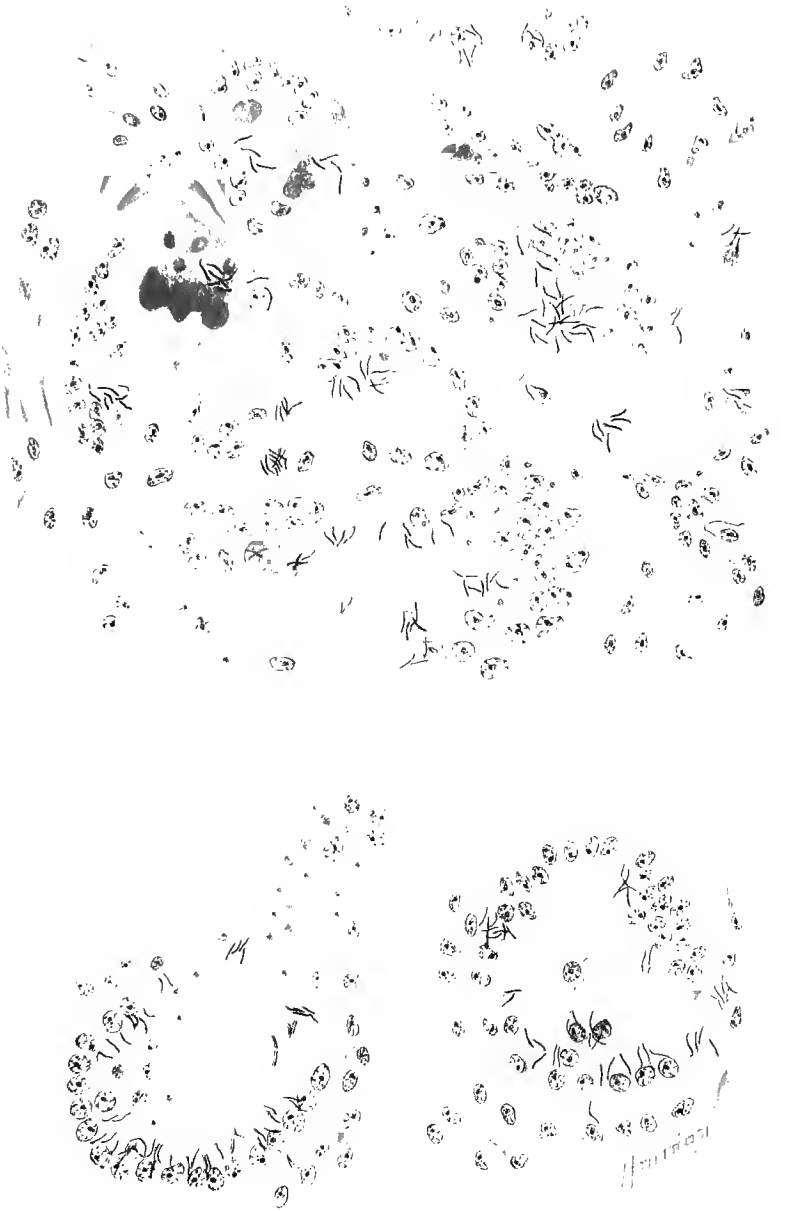
Avian Tuberculosis.—Robert Koch, in 1882, was of the opinion that avian and mammalian tuberculosis were the same disease and due to an identical microörganism. Later, however, he withdrew from this belief, and the preponderance of evidence today is that these two types of tuberculosis are not absolutely identical, but that they are in some important respects dissimilar diseases, due to two different varieties of the tubercle bacillus. Avian tuberculosis occurs among chickens and pigeons, also occasionally among geese and ducks. Natural infection occurs when healthy animals feed upon material soiled with the discharges of tubercular birds or when they directly eat some of the tubercular organs. Tuberculosis in birds most commonly occurs in the abdominal organs and the intestines and

the bacilli are spread through the feces. The liver and spleen are usually affected even if the intestines do not show any tubercular lesions. Frequently, however, the first pathologic changes are found in the intestines. Here the bacilli enter the mucosa, where they produce tubercles which soon degenerate and form ulcers. From the intestines the bacilli, through the blood current, enter the liver and later the spleen, lungs, joints and fasciæ of the tendons. The livers in birds dead of tuberculosis are generally much enlarged; the parenchyma cells may show a marked degree of fatty degeneration. The numerous tubercles vary in size from a millet seed to a pea and even larger. These nodules contain a dry, yellowish, grumous, caseous, sometimes chalky material. The mucosa of the intestine, when affected, shows yellow nodules and funnel-shaped tubercular ulcers. The mesentery may show larger nodules. The tubercles in birds differ somewhat from those in mammals, because the degeneration leads to the formation of a *material which is less granular and more hyaline* and dryer than that found in mammalian tuberculosis. Nocard and Leclainche describe the degenerated material in avian tuberculosis as a detritus with hyaline blocks, both infiltrated by an *amyloid material.* Such amyloid material is also found, according to Kitt, infiltrating the intestinal wall. Tubercles in ordinary fowls generally do not show many giant cells, few lymphoid cells, and mostly epithelioid cells and fibroblasts; but there appear to be very numerous giant cells in tuberculosis of guinea-fowl (Numida meleagris). In tuberculosis of the liver in these birds, as observed by the author, the giant cells are either round or oval, or they form irregular, apparently syncytial masses with nuclei irregularly grouped in the middle. Tubercular lesions are also frequently seen in fowl in the abdominal lymph glands and on the visceral layer of the peritoneum. Joint and bone tuberculosis are also common and lead to the formation of considerable dry caseous masses; the cartilages and the epiphyses are thickened and corroded in joint tuberculosis. Tuberculosis of the lungs is less frequent in fowl than tubercular affections of the organs named above. Tubercle bacilli are generally found in large numbers in the tubercular lesions of fowl.

Tuberculosis of Parrots.—While quite frequently observed, this is not a true avian tuberculosis, but an infection of these birds with human tubercular material. They generally contract the disease directly or indirectly from the sputum of consumptive persons. The portal of entrance of the virus infecting parrots is generally found in the skin of the head or the mucosa of the mouth, nose, or eyes. In advanced cases the abdominal organs, the joints, and bones are found affected, as in chickens in true avian tuberculosis.

Fish and Turtles.—A pathologic change with the formation of nodules due to the presence of acid-fast bacilli has also been described in fish and turtles.

PLATE IX



Avian Tuberculosis.

Section of the liver of a guinea-fowl.

QUESTIONS.

1. What is the characteristic anatomic lesion of tuberculosis? How can its formation best be studied, and why?
2. Describe the formation of an experimental tubercle.
3. Describe the formation of a polynuclear giant cell. What is an epithelioid cell? What is a lymphoid cell?
4. Describe the cellular elements of a young tubercle and their arrangement in the nodule.
5. Describe the new formation of vessels in the tubercle.
6. What is the evidence which shows that there occurs rapid cell proliferation after the entrance of virulent tubercle bacilli into the tissues of a susceptible animal?
7. Describe the relation between a multinuclear giant cell and the tubercle bacilli contained in it.
8. Does the tubercle contain fibrous connective tissue? What is a fibrous tubercle?
9. Does a tubercle contain polynuclear leukocytes, and if so, what is their mode of entrance?
10. What is meant by the caseation of the tubercle? What kind of process is caseation?
11. What is a fibrinous substance? To what factors are the peculiar physical properties of caseous material attributed?
12. What secondary changes occur in the caseous tubercle? to what are they due?
13. Describe fungous tubercular granulations.
14. What is a fibrocaseous tubercle?
15. What may be the fate of the giant cells in healing tuberculosis?
16. What is a solitary tubercle? Where are they generally found?
17. What is a hyperplastic tuberculosis?
18. What is meant by caseous infiltration? Where generally found?
19. What is a cold abscess? What a tubercular cavity? Describe its wall.
20. What is a mixed tubercular infection?
21. Describe a tubercular ulcer.
22. What is a miliary tuberculosis? How is acute, general miliary tuberculosis commonly, if not always, brought about?
23. What is the meaning of the term pearl disease? Why so called?
24. Give in general outlines the organs affected by tuberculosis in man.
25. Name the organs most generally affected in cattle, and describe the pathologic lesions as most commonly found in cattle.
26. Discuss tuberculosis in sheep, goats, cats, dogs, and horses
27. Describe tubercular lesions in chickens and pigeons. How is the disease generally spread among these domestic fowls?
28. What kind of tuberculosis do parrots generally contract?

CHAPTER XXX.

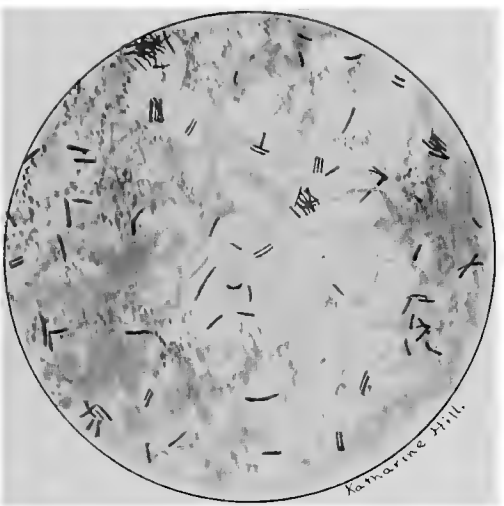
THE BACILLUS OF TUBERCULOSIS—TUBERCULIN TESTS—BOVO-VACCINE—THE INTERTRANSMISSIBILITY OF BOVINE AND HUMAN TUBERCULOSIS—AVIAN TUBERCULOSIS.

Morphology.—The tubercle bacillus is a rod-shaped organism, generally rather slender when found in human tuberculosis, more plump in the bovine type,[1] and commonly even more coarse in the avian type. It measures from 1.5 to 3.5 micra in length and 0.2 to 0.5 micron in breadth. It frequently occurs in pairs, the two individuals may or may not be in direct contact. When bacilli are numerous in sputum, pus, urine, or other fluid or semifluid tubercular products they often present themselves in parallel groups or clusters. The bacilli often have a beaded, granular appearance which is at times so pronounced that beginners gain the impression that they are dealing with short chains of streptococci. Under some conditions branching forms are seen; this proves that the organism of tuberculosis is perhaps not a bacillus, but more properly a member of the streptothricæ to which the ray fungus or actinomyces belongs. The bacilli frequently show in their interior unstained, oval, or slightly biconcave spaces, which were formerly mistaken for spores. It is now unanimously agreed that the tubercle bacillus does not form spores, because the forms which show these unstained spaces are not more resistant than the uniformly stained bacilli. This fact indicates the absence of the most important characteristics of the true spore—namely, its greater resistance to antiseptics, higher temperatures, drying out processes, etc. The tubercle bacillus is not motile and does not possess flagella. It does, however, possess a capsule containing a wax-like substance to which the bacillus owes two remarkable features—namely, its peculiar staining properties and its resistance, which is greater than that of most other non-sporulating pathogenic bacteria. It is also more resistant to drying out than most of the latter.

Staining Properties.—On account of the tough, tenacious wax-containing capsule, which surrounds the tubercle bacillus, it cannot, like most other pathogenic bacteria, be stained with the ordinary watery anilin stains. It requires a more intense stain, which either must act for a considerable time or be heated to intensify its action. After once having taken the stain, however, the tubercle bacillus holds it very firmly against the decolorizing action of dilute acids,

[1] The difference between the human and bovine types of the tubercle bacillus will be considered more fully under the discussion of the intertransmissibility of human and bovine tuberculosis.

PLATE X



Smear from a Tubercular Spleen of Cattle.

Zeiss hom. oil immersion objective 2 mm. Compens. ocular No. 6.

alcohol, etc. If, therefore, a pathologic product containing tubercle bacilli and other bacteria is treated with an intense hot staining solution and then subjected to the action of a decolorizing fluid and afterward counterstained with a contrast stain, the tubercle bacilli will have kept the first stain, while other bacteria have been dyed with the counterstain. In making use of this principle, tubercle bacilli can easily be stained and distinguished from other bacteria. There are, however, a few bacilli which act in a manner similar to the tubercle organisms These and the methods for their differentiation will be considered below. The bacilli of this group are known as *acid-fast bacilli.*

STAINING TUBERCLE BACILLI IN FLUID AND SEMIFLUID MATERIAL. —*Reagents Required*:

(*a*) Ziehl's Carbol-fuchsin.

1 Carbolic acid, 5 c.c.
2. Distilled water, 100 c.c.
3. Basic fuchsin crystals, 1 gram.
4. Absolute alcohol, 10 c.c.

Dissolve 1 in 2 and 3 in 4 and mix.

(*b*) 10 per cent. watery solution of nitric acid.

(*c*) 95 per cent. alcohol.

(*d*) Ordinary watery solution of methylene blue.

Steps in Staining, Decolorizing, Counterstaining.—

1. Prepare a cover-glass from suspected material, preferably selecting some of the cheesy flocculi, as they are found in sputum, pus, etc. After such material has been spread on a cover-glass, allow it to become air dry, and fix by passing three times through a flame.

2. Apply to the cover-glass, held in a suitable forceps, enough of Ziehl's carbol-fuchsin so that the whole surface is well covered; now hold high over a small flame until the staining solution boils. Then set it aside for a few minutes to permit the hot staining solution to act well.

3. Pour off the hot staining solution and wash the cover-glass in ordinary tap water or in distilled water.

4. Dip rapidly into the 10 per cent. watery nitric-acid solution, and at once wash freely in 95 per cent. alcohol. Continue washing the cover-glass, which is held in the forceps, in alcohol until apparently all of the red stain has been washed out, *i. e.*, until the cover-glass again appears almost unstained.

5. Dry between filter paper and for three or four minutes apply a thin watery solution of methylene blue.

6. Wash in water, dry between filter papers, mount on a slide in the usual manner.

Result of the Procedure Employed.—The tubercle bacilli are stained red; all other bacteria, yeast cells, etc., and cell nuclei are stained blue.

Precaution.—The procedure of staining tubercle bacilli and finding them subsequently, if they are present, is very easy, provided it is done carefully and a few precautions are observed. In the first place it is well to pour the suspected sputum or pus into a flat vessel, for instance a Petri dish, and to place it on a dark background. This makes it possible more easily to pick out with the platinum loop the small flocculent tubercular masses often found in tubercular material. In heating the carbol-fuchsin solution to boiling it must not be allowed to evaporate down too much, because then precipitates may be formed on the cover-glass. After having poured the hot stain off the cover-glass, washing in water is necessary, because if it is dipped directly into the dilute nitric acid the latter and the carbolic acid of the stain may form a smeary substance on its surface. The cover-glass must never be allowed to remain in the 10 per cent. nitric-acid solution long; it should just receive a dip. If after prolonged washing in alcohol, however, a good deal of red remains in the cover-glass preparation a second dip in the dilute nitric acid solution is not only permissible, but indicated. The watery methylene-blue solution should not be too concentrated, nor should it act too long, because if it does and there is much cellular material in the preparation an intense blue stain may cover up a small number of red-stained tubercle bacilli. When staining for tubercle bacilli in perfectly fluid media, such as, for instance, urine, the fluid is first centrifuged, the supernatant clear liquid decanted off, and the cover-glass preparation made from the sediment, which is then treated, stained, decolorized, etc., in the usual manner.

Biedert's Sedimentation Method.—When tubercle bacilli have *not* been found in suspected sputum or tenacious caseous material the material may be liquefied in order to centrifuge it and to increase the chances of finding the bacilli if only a few are present. The method then best used is Biedert's sedimentation method; its steps are as follows:

1. Place about a tablespoonful of the suspected material in a porcelain evaporating dish and add twice the amount of distilled water. Mix well by prolonged stirring with a glass rod.

2. Place the evaporating dish over a small flame and heat to boiling; continue stirring constantly, and while doing so add gradually ten drops of a 10 per cent. watery solution of caustic potash. Continue stirring and boiling until the mixture has become entirely fluid and has lost its tenacious, stringy character.

3. Cool and centrifuge; pipette off the supernatant fluid and preserve the sediment.

4. Take some of the original untreated material and spread on a cover-glass; then add some of the sediment and rub it up well with the material on the cover-glass.

5. Air dry, fix and stain, decolorize, etc., as usual.

This method often makes it possible to find few tubercle bacilli

which have escaped the ordinary procedure of making one or more cover-glass preparations.

STAINING TUBERCLE BACILLI IN PARAFFIN SECTIONS.—1. Sections cut as thin as possible are placed on clean slides with Meyer's egg-albumen mixture; they are then slightly heated over a small flame to melt the paraffin and to coagulate the fixing albumen Place directly in xylol to dissolve out the paraffin and then wash out the xylol in absolute alcohol

2. Place in a beaker containing Ziehl's carbol-fuchsin, and heat over a small flame until the staining solution begins to steam (do not heat to boiling, as this may damage the tissue). Leave in the hot staining solution for ten or fifteen minutes.

3. Wash well in water, then dip rapidly into a 20 per cent. watery solution of nitric acid, and immediately wash freely in strong (95 per cent.) alcohol until all the red color has been removed from the section. A second dip into 20 per cent. nitric-acid solution and another washing in the alcohol may be necessary.

4. Wash in water and stain for five minutes in dilute Loeffler's alkaline methylene blue (1 part of the stain to 2 parts of distilled water).

5. Decolorize by washing first in 95 per cent. alcohol, then in absolute alcohol until most of the blue stain has been washed out again, *i. e.*, until the nuclei only have retained the blue stain.

6. Clear in xylol, dry with filter paper.

7. Mount in Canada balsam.

In order to obtain a good, clear, nuclear stain, and to remove the excess of blue properly, it is necessary to look with a low-power lens at the slide after it has been in the xylol and before it is dried and covered with Canada balsam. The slide still wet with xylol is placed on the stage of the microscope and studied. If the section is still too blue, and if the nuclei have not been well differentiated, the slide must be returned to the absolute alcohol and again freely washed until a repeated microscopic examination shows that the desired effect has been obtained; then it may be mounted permanently and be examined with oil-immersion magnification for the red-stained tubercle bacilli.

STAINING TUBERCLE BACILLI IN CELLOIDIN SECTIONS.—1. Sections cut as thin as possible are stained rather lightly for three to four minutes in alum hematoxylin.

2. Wash in water.

3. Leave in warm (not hot) carbol-fuchsin solution for twenty to thirty minutes.

4. Wash in water.

5. Decolorize in acid alcohol[1] one-half to one minute.

6. Wash in several changes of water to remove every trace of acid so that the hematoxylin stain later shows a bluish color again.

[1] Acid alcohol is composed of hydrochloric acid, 1 c.c., and 70 per cent. alcohol, 99 c.c.

7. Wash well in 95 per cent. alcohol until all fuchsin has been dissolved out. This may require several changes of fresh alcohol.
8. Anilin oil one-half minute.
9. Several changes of xylol.
10. Mount on a slide in Canada balsam.

Cultural Properties.—The tubercle bacillus cannot be readily cultivated directly from sputum or caseous or purulent discharges containing it because it is not present alone, but mixed with other bacteria. Since the tubercle bacillus grows very slowly in artificial culture media the other bacteria multiply much more rapidly, and, in fact, overgrow it, making it difficult if not impossible to find it. It is certain that it can hardly ever be obtained in pure culture by the ordinary method of inoculating tubes directly or pouring plates. The usual method, therefore, of obtaining pure cultures consists in inoculating the tubercular material either subcutaneously or intraperitoneally into guinea-pigs. This highly susceptible animal develops first a local tuberculosis of the regional lymph glands and later a general tuberculosis. If such an infected guinea-pig is killed eight to ten weeks after the infection the proper tubercular material for obtaining pure cultures can be procured.

The procedure is best carried out as follows: The animal is chloroformed to death and then stretched out on a board with its four legs tied to four nails. The abdomen is shaved and sterilized and a median incision is made from the sternum to the symphysis. This is done with a sterile knife and in such a manner that the peritoneum is left unopened. The skin is then flapped back on both sides. The peritoneum is now opened in the middle line with fresh sterile instruments (scissors or knife) and held open with sterile forceps on both sides by an assistant or fastened to the side with suitable tacks. The operator now removes, with sterile forceps and scissors, some tubercular abdominal glands and the whole or part of the spleen. These tissues are immediately placed in a covered sterile glass dish (a not too shallow Petri dish will answer very well). The material so obtained is then divided, with all aseptic precautions, into small pieces, and portions from the interior of tubercular glands, or from tubercles in the spleen, are brought into tubes containing the proper culture media, where they are left either undisturbed on the surface or rubbed over it with a strong platinum loop. Suitable media for cultivating the tubercle bacillus are sterile coagulated cattle-blood serum (Koch), dog's blood serum obtained sterilly (Smith), eggs in which the white and yellow have been mixed and the mass subsequently distributed to test-tubes (Dorset), and various other media. After inoculation the tubes must be protected against evaporation, as it is necessary to keep them in the incubator at blood temperature for several weeks. This is done either with rubber caps, ground-glass stoppers, or by closing them with paraffin. If there is enough moisture in the tubes from the beginning the microörganism will grow without

air-tight closure of the tube. The growth, however, is very slow when the culture media have been inoculated from infected animals. In later successively transplanted generations the growth is somewhat more rapid and the bacilli are not so selective as to the medium in which they grow; after the first generation, for instance, they grow well on glycerin agar. In the first generation, growth generally is not easily recognizable with the naked eye until ten to fourteen days after inoculation. The multiplying bacteria form a dry, lusterless, granular, more or less wrinkled growth, slightly yellowish in color. Growth takes place best at blood temperature, and does not occur below 30° C. or above 42° C. The tubercle bacillus is a strict parasite, which does not find in nature the conditions to grow as a saprophyte. Tubercle bacilli have been found in the external world only where they have been spread by the discharges of tubercular persons or animals.

Toxic Effects.—These depend upon two principal factors, namely, (1) the metabolic products of the organism formed during its growth and multiplication in the invaded tissues of susceptible animals, and (2) the poisonous substances contained in the body of the bacterium. The latter substances, even after the bacillus is dead and can no longer multiply, cause local abscesses, necrosis, caseation, marasmus, and elevation of temperature. As has been shown by a number of authors, including Maffucci, Prudden, Hodenpyle, Strauss, and Gamalia, dead bacilli injected in small but sufficient quantities may produce tubercles with giant cells, but the process remains localized and tends to heal.

Resistance.—Tubercle bacilli in moist material are probably soon destroyed by the putrefactive bacteria and the changes they bring about. This was, at least, the view generally held formerly, but there are now on record some observations which show that this is not invariably the case. If tubercular material is placed in water the tubercle bacillus may remain alive for a considerable time. The bacillus is very resistant to drying out, and may remain alive a long time in desiccated tubercular material, such as sputum, pus, and other discharges; the average is about three months. Direct sunlight kills the organism if it is spread out in a thin layer, within a few minutes, but diffuse daylight requires five to seven days. Strauss found that glycerin bouillon cultures with an abundant growth were killed if exposed to direct sunlight for two hours Severe cold has no effect upon the bacillus. According to the figures given by Cornet and Meyer in their summary, and obtained from a *résumé* of the considerable literature upon the subject, the following periods of time and temperature exposures kill the tubercle bacillus in the moist state:

Four to six hours heated at 55° C.
One hour heated at 60° C.
Ten to twenty minutes heated at 70° C.
Five minutes heated at 80° C.
One to two minutes heated at 90° to 95° C.

In order safely to kill all tubercle bacilli contained in tubercular sputum it is necessary to boil it for five minutes at 100° C. The bacilli are very resistant to dry heat and can withstand 100° C. for one hour. Corrosive sublimate cannot be safely used to disinfect sputum, pus, caseous material, etc., because a peripheral coagulation of the albumen prevents its penetration into the interior of the masses containing the bacilli. Carbolic acid (5 per cent.) added to tubercular material in liberal quantities kills the organism within a few hours. Formalin is not trustworthy as a disinfectant for tubercular albuminous material.

Tubercle Bacilli in the Circulating Blood.—In much advanced cases tubercle bacilli have occasionally been found in the circulating blood, but this is an exceptional occurrence. They evidently do not multiply in the blood and are filtered out very soon after entering it. It therefore occasioned considerable surprise when Rosenberger claimed that he had been able to show that tubercle bacilli are present in the blood even in the most incipient cases. In other words, Rosenberger claimed that tuberculosis was a *bacteriemia*. However, it has been shown by McFarland and his co-workers, and by Ravenel and Smith, that this statement is incorrect for human tuberculosis. Schroeder, Colton, and Mohler examined the blood of 50 tubercular cows, not merely by staining methods, but by the inoculation of 135 guinea-pigs, and found that Rosenberger's claim was entirely unsubstantiated; tuberculosis accordingly is a bacteriemia neither in man nor cattle. Rosenberger in his painstaking work became the victim of a deceptive acid-fast bacillus often found in distilled water used in laboratories.

TUBERCULIN TESTS—THE DIAGNOSIS OF LATENT TUBERCULOSIS IN CATTLE.

Koch's Old Tuberculin.—Koch's old tuberculin, also known as Tuberculin Original, or Koch's O. T., is prepared as follows: A veal bouillon, containing 3 to 5 per cent. glycerin and the usual amounts of common salt ($\frac{1}{2}$ per cent.) and pepton (1 per cent.), having been made slightly alkaline and kept in a flask, is inoculated on the surface from a pure culture of tubercle bacilli. The flask is then kept in the incubator at blood temperature from three to six weeks. During this time a thick, dry, crumpled, whitish layer forms on the surface. When this is well developed stains are prepared from it, and if the culture is found to be pure and uncontaminated it is evaporated on a water bath at a temperature of 70° to 80° C. down to one-tenth of its original bulk. The thick brown liquid so obtained, containing from 30 to 50 per cent. glycerin, is first filtered through chemical filter paper and then through a Chamberland or Pasteur filter. The product of these manipulations is known as

crude tuberculin; it is a fairly stabile product, and can be kept in a refrigerator for a long time. Before use it is *diluted with nine times its bulk of a sterile ¼ per cent. watery carbolic-acid solution.* This old tuberculin is today still considered the best preparation for diagnostic use in man and cattle.

A good tuberculin should kill in a minimal dose of 0.5 c.c. a guinea-pig which has been infected with tuberculosis three to four weeks before the tuberculin is tested. Death should occur within six to thirty hours. Sometimes tuberculins are much stronger and kill a guinea-pig previously infected (three to four weeks) in doses of 0.1 c.c. to 0.05 c.c. Guinea-pigs infected eight to ten weeks previously will be killed in centigram doses of a good strong tuberculin.

In testing cattle with tuberculin for latent tuberculosis the following doses of the dilute tuberculin are used:

For large bulls or steers, 4 c c
For large cows, 3.5 c.c.
For medium-sized cows and steers, 3 c.c.
For heifers and young bulls, one to two years old, 2 c.c.
For calves under one year, 1 c.c.
For sheep and goats, 0.5 to 1 c.c.
For pigs of various ages, 1 to 3 c.c.
For pigs under four months, 1 c.c.
For pigs from four to nine months, 1.5 to 2 c.c.
For pigs from nine to sixteen months, 2.5 c.c
For pigs above eighteen months, 3 c.c.

Different brands of tuberculin furnished by government and private institutions generally state the exact doses for various animals at various ages.

Federal Government Directions for Inspecting Herds with the Tuberculin Test.—Inspections should be carried on while the herd is stabled. If it is necessary to stable animals under unusual conditions or among surroundings that make them uneasy and excited, the tuberculin test should be postponed until the cattle have become accustomed to the new conditions. The inspection should begin with careful physical examination of each animal. This is essential, because in some severe cases of tuberculosis no reaction follows the injection of tuberculin on account of saturation with toxins, but experience has shown that these cases can be discovered by physical examination. The latter should include a careful examination of the udder and of the superficial lymphatic glands and auscultation of the lungs.

Each animal should be numbered or described in such a way that it can be recognized without difficulty. It is well to number the stalls with chalk and transfer these numbers to the transfer sheet, so that the temperature of each animal can be recorded in its appropriate place without danger of confusion. The following procedure has been used extensively and has given excellent results:

(*a*) Take the temperature of each animal to be tested at least twice at intervals of three hours before tuberculin is injected.

(*b*) Inject the tuberculin in the evening, preferably between the hours of 6 and 9 P.M. The injection should be made with carefully sterilized hypodermic syringes. The most convenient point for injecting is back of the left scapula. Prior to the injection the skin should be washed carefully with a 5 per cent. solution of carbolic acid or other antiseptic.

(*c*) The temperature should be taken nine hours after the injection, and temperature measurements repeated at regular intervals of two to three hours until the sixteenth hour after the injection.

(*d*) When there is no elevation of temperature at this time (sixteen hours after injection) the examination may be discontinued, but if the temperature shows an upward tendency, measurements must be continued until a distinct reaction is recognized or until the temperature begins to fall.

(*e*) If the reaction is detected prior to the sixteenth hour the measurements should be continued until the expiration of this period.

(*f*) If there is an unusual change of temperature of the stable, or a sudden change in the weather, this fact should be recorded on the report blank.

(*g*) If a cow is in a febrile condition tuberculin should not be used, because it would be impossible to determine whether, if a rise in temperature occurred, it was due to the tuberculin or to some transitory illness.

(*h*) Cows should not be tested within a few days before or after calving, for experience has shown that the result at this time may be misleading.

(*i*) The tuberculin test is not recommended for calves under three months old.

(*j*) In old, emaciated animals and in re-tests use twice the usual dose, for these animals are less sensitive.

(*k*) Condemned cattle must be removed from the herd and kept away from those that are healthy.

(*l*) In making postmortems the carcasses should be thoroughly inspected and all the organs should be examined.

Reaction after Tuberculin.—The characteristic reaction which occurs when an animal affected with tuberculosis receives an injection of good active tuberculin in the proper dose (see above) is the following:

The temperature begins to rise gradually six to twelve hours after the injection; twelve to twenty-one hours after the injection it reaches its maximum; between the twenty-fourth and fortieth hours it returns to normal. There is, however, during this interval between the twenty-fourth and fortieth hour not infrequently a second but more moderate rise. In rare cases the first rise may begin before the sixth or after the twelfth hour, but these are rather exceptional

cases. The respiration and pulse often become increased in relation to the rise in temperature, at other times these physiologic functions show no marked changes from the normal, Sometimes about the sixth to the eighth hour after the injection the animal suffers from some weakness and lack of appetite. Trembling of the muscles may also be observed. The secretion of milk during a positive reaction becomes reduced, generally between 3 and 8 per cent., exceptionally as high as 13 per cent. There is no direct relation between the intensity of the reaction and the intensity of the tubercular process, except that much advanced cases of tuberculosis in weakened animals give a weak reaction.

The tuberculin test has, as a rule, no lasting ill effect, except in very weak animals with advanced tuberculosis, where the test may be followed by a more or less permanent rise in temperature and a more rapid course of the tubercular process. Healthy animals do not react or only very slightly to the ordinary diagnostic doses or to even larger doses of tuberculin; this is also true of animals suffering from other diseases.[1] It should also be noted that a positive reaction can only be expected after the tubercular infection has existed for a certain minimum time. Nocard showed by experiments that at least two weeks must elapse between the time of infection with moderate doses of tubercle bacilli and the time of the first appearance of a positive tuberculin test. McFadyean found a positive reaction in eight days after very large doses of infecting bacilli. After natural infections it requires doubtless a much longer time before a positive test can be obtained. The limits given above all refer to infection by injection. According to Nocard and Rosignol, infection through the intestinal tract is followed by a positive tuberculin reaction after thirty-two to forty-eight days; while infection by inhalation leads to positive reactions after nineteen to thirty-two days.

Nocard regards a rise of 1.4° F. as insignificant; one from 1.4° to 2.5° F. (always provided that it occurred in the typical gradual manner) as suspicious and requiring re-testing at the end of a month; while a rise of 2.5° to 5.4° F. absolutely indicates the presence of a tubercular process in the animal tested.

The Eighth International Veterinary Congress (1905, Budapest), adopted the following resolutions in regard to the tuberculin test:

1. The preparation and supply of tuberculin should be controlled by the State.

2. No animal whose temperature exceeds 39.5° C. (103° F.) is a fit subject for the tuberculin test.

3. A rise of temperature to above 40° C. (104° F.) in any animal whose temperature at the moment of injection was below 39.5° C. (103° F.) is to be regarded as a positive reaction.

[1] The tuberculin test is, of course, not applicable in the presence of high fever no matter to what it may be due.

4. Any rise in temperature between 39.5° C. (103° F.) and 40° C. (104° F.) must be regarded as of doubtful significance; animals exhibiting such require special study.

The question of how soon an animal reacting positively will again react has been studied experimentally by a number of investigators. According to Nocard's experiments on 24 cows reacting positively upon the first test, 33 per cent. reacted positively to another test made twenty-four to forty-eight hours after the termination of the first test; 50 per cent. positively after eight days; 60 per cent. positively after two weeks, and 100 per cent. after one month. According to the experience in the Prussian Sea Quarantine Station, 100 per cent. react positively in a second test made almost immediately after the first has completely disappeared, but five times the amount of the first test dose must be used. According to Valle the double dose of the first test dose will give a positive result when used thirty-six to forty-eight hours after the termination of the first reaction. The maximum temperature, however, is reached much earlier in the second test and the fall in temperature is more rapid than in the first test.

The accuracy of the conclusions drawn from the test varies according to the good judgment of those who make the test. According to Bang, under satisfactory conditions the positive diagnosis upon the basis of the test is found correct in 96 per cent. According to Jensen's statistics 90.8 per cent. of tubercular animals out of a total of 468 gave a positive result. Ever's statistics of 563 cases later examined by postmortem examinations showed a correct interpretation of a positive test in 86.9 per cent. The recent experience in the United States with the tuberculin test of cattle has been even more favorable than the earlier observations in Europe. Melvin reported that of 24,784 head of cattle tuberculin-tested in 1907-08 with a positive reaction, 24,387, or 98.39 per cent., upon being slaughtered were found to be tubercular. Nocard and Hutyra and Marek state that animals affected with echinococcus or actinomycosis never react positively to the tuberculin test.

Ophthalmo-tuberculin Reaction.—Wolf-Eisner and Calmette almost simultaneously and independently introduced another form of tuberculin reaction into medical practice. It consists in the instillation of one or two drops of a tuberculin solution prepared in a special manner from the watery solution of an alcoholic precipitate of a killed tubercle bacilli culture into the conjunctival sac near the inner canthus of the eye. The test has been found quite accurate in man, and several observers have tried it on cattle. Bailliart, reporting to the Sixth International Tuberculosis Congress in Washington, stated with reference to the ophthalmo-tuberculin test in cattle: "The ophthalmo-reaction is a diagnostic procedure which is usually without danger if it is applied only to eyes free from tubercular lesions of any kind. It is sometimes followed by mild and

transitory accidents. Very often the reaction is doubtful. In cattle, doubtful cases, owing to the difficulty of examination, must be regarded as negative. In these animals a simple (primary) ophthalmo-reaction is a very unreliable procedure and cannot take the place of inoculation with tuberculin. A secondary ophthalmo-reaction gives very much more trustworthy results." White and McCampbell, reporting on the same subject to the same Congress, however, place more confidence in the primary test, but they likewise do not consider it of much value in the diagnosis of tuberculosis in cattle. In some cases the reaction is very slight, in others pronounced congestion with profuse exudates is noted. They were inclined to rely primarily on the result of the first instillation of tuberculin. In the majority of animals tested the reaction increases in its intensity with each subsequent instillation of tuberculin. This fact indicates the development of a local hypersusceptibility, or anaphylaxis, associated with a partial immunity.

Pirquet's Cutaneous Tuberculin Test.—This consists in the vaccination with a few drops of tuberculin on the arm, and has been extensively used in children but very little in animals. The same is true of the so-called *dermo-reaction*, where a tuberculin-lanolin ointment is rubbed into the skin. These two methods, like the ophthalmo-tuberculin test, are of no great value in the detection of latent tuberculosis in domestic animals.

IMMUNIZATION AND PROTECTIVE INOCULATION.

Bovovaccine.—Starting from the viewpoint that the bacillus of human tuberculosis, derived from the sputum of tubercular patients, is not very virulent for cattle, von Behring has worked out a method of protective and immunizing inoculation. Since tubercle bacilli of human derivation, even apart from those comparatively not numerous cases of infection by bacilli of the bovine type, vary a good deal in their effect upon cattle, it is not a matter of indifference what stem of human tubercle bacilli is selected for the preparation of a vaccine to be used on bovines. Von Behring, after many preliminary experiments, selected a culture of a low degree of virulency for cattle which had never led to any untoward accidents. From transplants of this original and extensively tested culture all his bovovaccine has been prepared. The experience of von Behring and his co-workers and of those who have tried his method and vaccine, like Hutyra, a French commission and others, has been very favorable both as to the harmlessness of the procedure and as to the protection which it affords against natural infection or artificial inoculation with the bovine tubercle bacillus. The bovovaccine is prepared from a four to six-weeks-old glycerin bouillon culture of the selected human tubercle bacillus. The culture is filtered and the bacilli remaining on the filter are dried *in vacuo* over sulphuric acid for twenty-four

hours. The mixture is then somewhat triturated and definite amounts are placed in small glass tubes. These either contain five or twenty immunity units (J. E.).[1] One immunity unit (J. E.) is equal to 0.004 gram dry tubercle bacilli. It is the dose used for the first inoculation of young cattle, while a dose of 0.02 gram or 5 immunity units is employed for the second inoculation, which follows the first after an interval of twelve weeks. The vaccine is prepared from the dry bacilli by mixing the contents of the tubes with sterile physiologic salt solution, so that 5 J. E. are mixed with 10 c.c.; 20 J. E. with 40 c.c. salt solution. For the first inoculation 2 c.c. of the salt solution bacilli emulsion are used; for the second inoculation after twelve weeks 10 c.c. of the emulsion. Only healthy animals with a normal temperature should be inoculated. The inoculation is made into the jugular vein. It is hardly necessary to point out that the mixing of the dry bacilli and the injection of the emulsion must be done under all possible aseptic precautions.

Weber and Titze who have employed the von Behring method of cattle immunization believe that the immunity conferred by it does not last over two years. Smith vaccinated 35 calves, weighing from 58 to 284 pounds, with seven different human and three attenuated bovine cultures, and from his experimental work he draws the following conclusions:

1. Vaccination of calves with the human type of the tubercle bacillus is harmless. Cases in which injuries are said to have resulted from it may have been due to other concomitant affections, among which pneumonia is probably the most common. Persons trying vaccination should first assure themselves that the culture they intend to use belongs to the human and not to the bovine type of the bacillus.

2. Vaccination with the human type of bacillus leads to a relatively high resistance to fatal doses of the bovine bacillus.

3. Vaccination with carefully tested attenuated bovine bacilli may be as efficacious even in a single injection as the double vaccination with human bacilli. Such vaccination may be less dangerous to man than when human bacilli are used.

4. The immunity conferred by vaccination, as hitherto practised, does not appear to be satisfactory as regards degree or duration. More evidence is needed with regard to these points. The herds of large public institutions are well adapted to decide these questions if vaccination is thoroughly applied, and the animals supervised by properly trained men.

5. Insufficient immunity following vaccination may prove dangerous in giving rise to mild cases, after ordinary exposure in infected herds, which tend to discharge tubercle bacilli from small foci in the lungs.

[1] The term immunity unit (J. E.) as used in connection with the bovovaccine of von Behring differs in meaning from the identical term as used to express the immunizing value of diphtheria nr tetanus antitoxins.

6. The immunity acquired by two vaccinations with human bacilli should be fortified by a subsequent injection of attenuated bovine bacilli.

7. Investigations should be made looking toward the selection, by the injection of attenuated bovine bacilli, of races or breeds of cattle which posses naturally a high degree of resistance to tuberculosis. The capacity of different breeds to acquire a high degree of immunity should also be investigated.

8. The survival of human and bovine bacilli in the lungs and udders of calves vaccinated intravenously with them should be more definitely determined.

9. Vaccines may be easily and cheaply prepared in the form of suspensions in fluids ready for injection. The length of time during which suspensions maintain their highest efficiency remains to be determined.

Prophylaxis and Eradication of Tuberculosis among Domestic Animals.—The recognition of the fact that tuberculosis is an infectious disease, due to a specific bacillus or to several varieties of a single species, has led to effective measures to prevent the spread of the disease, particularly after the extensive and excellent work of Cornet had shown that the tubercle bacillus is not ubiquitous but only present where it is disseminated by tubercular persons or animals. Human tuberculosis has been reduced particularly by such measures as the destruction of the tubercular sputum, the prevention of its broadcast dissemination, the isolation to a certain extent of tubercular persons, the avoidance of overcrowding of human residences, and the proper use of direct and diffuse sunlight as a disinfectant against the tubercle bacillus. Efforts have also been made on a large scale to prevent tuberculosis and limit its spread among the meat and milk-producing animals. These efforts are justified and demanded imperatively, not merely because there is a certain amount of danger of the spread of tuberculosis from domestic animals to man (though the principal danger among mankind is the infected person), but also because tuberculosis among domestic animals is a constant source of great national loss, and its eradication would be a great purely economic gain apart from all hygienic considerations. An elaborate plan for finally stamping out tuberculosis among cattle has been devised by Bang. The method consists in a careful clinical examination of all cattle and subjection of the animals to the tuberculin test. Those animals which already show well-marked clinical evidence of tuberculosis, particularly of the lungs, intestines, uterus, and udder, are separated from the others and slaughtered as soon as possible. The apparently healthy animals which do not react to the tuberculin test are likewise placed by themselves in a separate class. If possible they are kept in a separate barn, but if this is impracticable, they are at least divided from the others by a partition wall in a space

with separate entrance. After the removal of the evidently tuberculous animals and those which have reacted to the test the place where the healthy animals are segregated should be thoroughly disinfected so that any remaining tubercle bacilli may be destroyed. The more thorough the segregation, even to the extent of having their own separate attendants, the better. Animals which have reacted to the tuberculin tests but are otherwise in good condition may be used for breeding purposes. The calves, however, should be separated from their tubercular mothers within twenty-four hours after birth. They must then be brought up either on the raw milk of healthy cows or, if on their mother's milk, it must be pasteurized at a temperature which will safely destroy the tubercle bacilli. If any of the calves show diarrhea they must be separated immediately from the other young animals. As soon as possible the calves themselves are subjected to the tuberculin test and the few which react positively must be separated from the healthy ones. From time to time the healthy cows must be re-tested with tuberculin so as to forestall a clandestine appearance and spread of tuberculosis among the non-infected stock. If this method is carried out persistently, intelligently, and carefully it will, in a few generations, very much reduce tuberculosis in a herd and will eventually lead to its ultimate eradication. The results in Denmark, under Bang's personal supervision, have been very excellent. Hutyra and Marek report brilliant results from the Hungarian State Cattle Breeding Farm. In the spring of 1898 the first tuberculin test of 329 cows showed 44.8 per cent. infected; a later test of 647 head showed 26.6 per cent. infected animals. In the fall of 1901, 1042 head showed 3.1 per cent. infected, and the test in 1903, of 1132 animals, showed only 1.8 per cent. infected. In other words, during five years the number of animals, without outside additions, had increased very much, and tuberculosis had decreased 88 per cent. These are, indeed, encouraging figures.

Prevention of Tuberculosis among Swine.—Among the more important precautions to prevent the spread of tuberculosis among swine are the following: The animals should not be allowed to feed after tubercular cattle nor to devour the carcasses of cows dead from tuberculosis, and skimmed milk from non-tested cows should not be fed to hogs until it has been properly pasteurized or sterilized.

A community or State which does not try to prevent the spread of tuberculosis and make proper attempts to stamp it out ultimately among its food and milk-producing domestic animals by proper equitable regulations, ordinances, and statutory laws is sorely lacking in doing its duty toward its citizens, the more so because there are States where such laws are in existence. This must necessarily operate in such a manner that the non-protected territory will become the dumping ground for tubercular animals condemned elsewhere.

THE IDENTITY OR NON-IDENTITY AND INTERTRANSMISSIBILITY OF TUBERCLE BACILLI FROM VARIOUS SOURCES.

Bovine Tuberculosis.—When Robert Koch, in 1882, made public his work in connection with the discovery of the tubercle bacillus he believed that tuberculosis of man and animals was an absolutely identical disease and that tubercle bacilli, no matter from what source they were derived, were virtually identical in each and every respect. Smith, in 1896, was the first to call attention to the fact that tubercle bacilli from human tubercular sputum and those derived from bovine tubercular lesions present certain differences in their morphology, biology, and virulency toward various animals. A more extensive communication concerning this work followed in 1898, and another report was published in 1905. Smith's work as to differences in virulency toward domestic animals has since been confirmed by Dinwiddie and others, and it has been established that tubercle bacilli from bovine sources are generally short, straight, and cylindrical in outline. At first they grow rather poorly on artificial media, are less influenced by variations in the composition of the medium, and tend to remain short when grown on artificial media for a number of generations. Bacilli derived from human tubercular sputum, on the other hand, are generally more slender from the beginning, often curved. They grow much more abundantly in first generations on artificial media and show a tendency to remain slender or to become so if they did not show this form in a decided manner from the beginning. Mohler and Washburn, in a comparative study of tubercle bacilli from varied sources, have come to the following conclusions as to a certain plastic variability in their morphologic features, and say:

"While certain peculiarities of growth, morphology, and pathogenesis are observed with a fair degree of constancy in bacilli of human origin, nevertheless these characteristics are not universal, and notable exceptions are observed which would confuse those who would attempt to establish their origin by means of such characteristics. A similar degree of constancy in the morphologic, biologic, and pathogenic characters of the bovine bacillus is generally noted; but a certain range of differences has been observed, which, though apparently more limited than for the human bacillus, is nevertheless suggestive of aberrant forms."

An important biologic difference in the action of human and bovine tubercle bacilli in changing the reaction of the culture medium is described by Smith as follows: "When flasks of glycerin bouillon in layers 1½ to 2½ centimeters deep are inoculated with scales of tubercle bacilli from glycerin-agar cultures the floating masses soon begin to expand, and after one or two months a complete membrane will have formed over the relatively clear bouillon. The bacillar masses which fall to the bottom at the outset increase but slightly in size." This

mode of surface growth of tubercle bacilli was shared by all mammalian races examined by Smith after they had acquired a certain saprophytic habit, and the process is well known to all who have cultivated tubercle bacilli for the preparation of tuberculin. If ordinary bouillon prepared from fresh beef to which 3 to 5 per cent. glycerin has been added is used, and if the acidity is made equivalent to about 2 per cent. of normal acid, phenolphthalein being the indicator, the reaction of the bouillon during the formation of the membrane will take one of two directions. It may approach the neutral point and reach an acidity or an alkalinity of about 0.1 to 0.2 per cent. when the membrane is complete and remain at this point, or else the acidity may diminish during the first months, increase again during the second, and fluctuate more or less if the observation is continued, but the reaction does not reach the neutral point. In the human cultures, on the other hand, the reaction curve also moves toward the neutral point, but soon swings back to a greater acidity.

The human type of tubercle bacillus has very slight virulency toward cattle. This fact may indeed be considered as established. It has been proved beyond question that human sputum bacilli used on cattle in doses in which bovine bacilli will produce general tuberculosis have, as an almost universal rule, practically no lasting effects whatever. Bovine tubercle bacilli are likewise more virulent than the human type toward guinea-pigs, rabbits, mice, rats, sheep, goats, pigs, dogs, equines, etc. Rabbits are particularly well adapted to show the difference in virulency between the bovine and human types when applied to the same species of animals. According to Weber, 0.001 gr. of bovine bacilli injected intravenously kills rabbits with the production of a general tuberculosis in three weeks, while the human type under the same conditions produces a very chronic slow form of tuberculosis only after months. The first extensive experiments showing that such is the action of human sputum bacilli toward cattle were made by Koch and Schütz. Upon the basis of these observations Koch, in 1901, before the British Congress on Tuberculosis appears to have taken the standpoint that the danger of transmitting bovine tuberculosis to man is practically nil. After 1901, however, it was ascertained, and Ravenel had, indeed, made some observations before this time, that tubercle bacilli of the bovine type, very virulent[1] to cattle, are found in certain tubercular lesions in man. Such bacilli, however, with a possible single exception (Arloing), have never been found in the sputum of tubercular patients, and since human sputum bacilli were exclusively used in the early experiments of Koch and Schütz the former was induced to draw conclusions which were too radical and which cannot be upheld at the present time. Koch, in 1908, at the Inter-

[1] Ravenel found such bacilli in the mesenteric glands of a child.

national Congress at Washington, had evidently modified his standpoint, which he then expressed as follows:

"1. The tubercle bacilli of bovine tuberculosis are different from those of human tuberculosis. 2. Human beings may be infected by bovine tubercle bacilli, but serious diseases from this cause occur very rarely. 3. Preventive measures against tuberculosis should therefore be directed primarily against the propagation of human tubercle bacilli."

Since 1901 a great number of experiments have been made in relation to the question of the difference in virulency between human and bovine tubercle bacilli, the possibility of infecting cattle with bacilli of human derivation, and presence or absence of bacilli of the bovine type in human tubercular lesions. Those who followed Koch and Schütz did not confine themselves to human sputum bacilli, but also chose tubercular material from other sources, and it was soon found that such material sometimes contained bacilli of the bovine type which are quite virulent to cattle. Kossel, Weber and Heuss, after Koch's and Schütz' experiments, examined 56 cases of human tuberculosis and found in 49 bacilli of the human type, in 5 bacilli of the bovine type, and in 2 bacilli of both types. The 5 cases in which they found bacilli of the bovine type were those of children under five years of age, 4 of which warranted the conclusion that the bacilli had gained entrance through the intestinal tract. The 49 cases in which bacilli of the human type only had been found included many forms of tuberculosis, such as tuberculosis of the lungs, lymph glands, bones, joints, genito-urinary tract, intestinal tract, peritoneum, and meninges, and general miliary tuberculosis. The tubercular subjects were of all ages, and among them 2 cases of tuberculosis of the peritoneum were found which anatomically were of the "Perlsucht" type, but in which the bacilli were not of the bovine type but the human kind.

Weber, in the article on "Tuberculosis of Man and Animals," in Kolle and Wassermann's *Manual of Bacteriology*, enumerates, including his own, 14 cases of tuberculosis in man, collected from literature, in which bacilli of the bovine type had been isolated beyond doubt. Since the publication of his report several further cases have been added to the list by Smith and Mohler and Washburn, and perhaps by some others, but it is safe to say that there are not many above 50 cases now on record, and none of these were cases of pulmonary human tuberculosis. It appeared to be the general consensus of opinion at the last International Congress on Tuberculosis that Koch's contention was correct and that no case of human pulmonary tuberculosis had yet been traced beyond doubt to the bovine tubercle bacilli, because the few cases reported as such were not beyond reasonable criticism. The cases of human tuberculosis shown to be caused by bacilli of the bovine type are, as appears, all traceable to the entrance of the bovine bacilli through the intestinal tract, probably

with highly contaminated milk. It must, therefore, be considered as established that man can be and is occasionally infected with bovine tuberculosis and that tubercular cattle, particularly cows, are not a negligible source of danger to man but one which warrants measures to prevent the spread of tuberculosis from cattle to man.

Avian Tuberculosis.—There are certain differences in the morphology and biology of the avian tubercle bacillus compared with the mammalian races. Microscopically it resembles, on the whole, the human type rather than the bovine, but very often it shows considerable pleomorphism, with numerous coarse, club-shaped, and branched forms. It grows more rapidly in artificial cultures than either the human or the bovine type, and develops, in ten to fifteen days, round, moist colonies, which grow rapidly and then form a continuous, dull-shining, wax-like, fatty film over the surface of the medium, which later becomes corrugated and assumes a decidedly yellow color. In its moist character cultures of avian tubercle bacilli vary greatly from the dry cultures of mammalian organisms. The latter do not grow at temperatures over 41° C., while the former still multiply at temperatures between 45° and 50° C. Guinea-pigs are quite resistant toward avian tubercle bacilli, but they sometimes contract the avian disease in artificial inoculation and die from it. Rabbits appear to be more susceptible to avian than to human tubercle bacilli. The horse, according to Nocard, is susceptible to avian tubercle bacilli in artificial infection. Chickens and pigeons are very susceptible and develop the typical picture of avain tuberculosis. These birds are generally not very susceptible to bacilli of human derivation, though some observers have had positive results in inoculation experiments, and it is also claimed that barnyard fowl have been infected from the sputum of tubercular patients. Straus and Wurz and Nocard, however, have fed chickens for a long time on tubercular sputum without producing the disease. Nocard implanted collodion sacs containing human tubercle bacilli in the peritoneal cavity of chickens, and succeeded in changing their morphologic and cultural characteristics and their pathogenicity for chickens so that they became much like the bacilli of avian tuberculosis. Mohler and Washburn investigated an outbreak of tuberculosis among the fowl of a large ranch in Oregon. This epizoötic seemed to extend to the swine of the same place, through feeding the hogs on the carcasses of fowl that succumbed to the disease. They found that young pigs could be infected with tuberculosis by being fed material from tuberculous chickens from this ranch, and that these avian bacilli, after a repeated passage through mammals, would produce typical lesions of tuberculosis in the latter.

From the various observations quoted above it appears that avian tuberculosis can be transmitted to mammals, and vice versa. It is, of course, an established fact that parrots in captivity are quite susceptible to tubercular infections from man.

QUESTIONS.

1. Describe the morphology of the tubercle bacillus?
2. Why have some authors classified the tubercle bacillus under streptothricæ?
3. Describe spore formation of the tubercle bacillus and its flagella.
4. Why is the tubercle bacillus difficult to stain?
5. What is an acid-fast bacterium?
6. What reagents are needed for staining the tubercle bacillus?
7. Describe the steps in staining the bacillus in sputum, pus, caseous material, etc.
8. Describe Biedert's liquefaction and sedimentation method for finding a few tubercle bacilli.
9. Describe the method of staining tubercle bacilli (*a*) in paraffin sections; (*b*) in celloidin sections.
10. Describe the method of obtaining a first generation of a pure culture of tubercle bacilli from tubercular pathologic material.
11. What are the media used for obtaining the bacillus in pure cultures?
12. Upon what do the toxic effects of the tubercle bacillus depend?
13. Discuss the resistance of the tubercle bacillus.
14. What disinfectants are efficient in the destruction of the tubercle bacillus? Which are inefficient?
15. Describe the preparation of Koch's old tuberculin used in making the tuberculin test in cattle.
16. What are the doses to be used on animals of various ages and sizes?
17. What is the standard of a good reliable tuberculin?
18. Describe the steps in making the tuberculin test in cattle.
19. Discuss the interpretation of the result of the test.
20. How soon will an animal react positively again after once reacting positively?
21. What is the accuracy of the tuberculin test in cattle?
22. What is the ophthalmo-tuberculin reaction? What is its value in cattle?
23. What is the von Pirquet test?
24. Discuss the preparation, use, and value of von Behring's bovovaccine.
25. What is the opinion of various investigators concerning this method of protective inoculation?
26. What has been the most successful single measure in limiting the spread of tuberculosis (pulmonary) in man?
27. Describe in detail the method of Bang for limiting the spread of tuberculosis among cattle and finally stamping it out entirely.
28. What measures should be taken to prevent the spread of tuberculosis among swine?
29. What are the differences in morphology between the so-called human type and the so-called bovine type of tubercle bacilli?
30. What are their cultural differences?
31. What is their difference as to acid production in glycerin bouillon?
32. What is the virulency of the human tubercle bacilli toward cattle?
33. What is the comparative virulency of the human and the bovine type toward rabbits?
34. What toward pigs, guinea-pigs, and equines?
35. What was the result of Koch and Schütz's inoculation experiments with human sputum bacilli on cattle?
36. Is bovine tuberculosis ever transmitted to man? If so, in what kind of cases of human tuberculosis has such transmission been found?
37. What percentage of human pulmonary tuberculosis can be traced to cattle?
38. What was the result of Kossel, Weber, and Heuss' researches and experiments with a variety of human tubercular material as to its human or bovine origin?
39. Have other cases of similar type been reported, and by whom?
40. Mention some of the differences between avian and mammalian tubercle bacilli, both morphologic and cultural.
41. Are avian and mammalian tuberculosis intertransmissible, and, if so, what do we know about it?
42. Describe Nocard's method of changing human tubercle bacilli into the avian type.

CHAPTER XXXI.

PSEUDOTUBERCULOSIS AND ACID-FAST BACILLI OTHER THAN THE TUBERCLE BACILLUS—RAT LEPROSY—CHRONIC BACTERIAL DYSENTERY (JOHNE'S DISEASE) IN CATTLE.

Pseudotuberculosis.—When, after the discovery of the tubercle bacillus, numerous investigators inoculated tubercular material, or what appeared to be such, into laboratory animals it was ascertained that these sometimes developed lesions which on first sight appeared to be tubercular, but which on careful examination were found not to contain the tubercle bacillus but other microörganisms. Observations of this kind multiplied, and it has become customary to classify such pseudotubercular pathologic changes due not to tubercle bacilli, but to entirely different organism as pseudotuberculoses. It was further found that such pseudotubercular infections occurred not only after artificial inoculations, but also spontaneously as laboratory epizoötics or among domestic and wild animals.

Preisz, after having discovered a bacterium of this kind, and after reviewing the work of others in this field, divided the bacillary pseudotuberculoses into the three following groups based upon the causative microörganisms:

I. Pseudotuberculoses due to the Bacillus pseudotuberculosis rodentium (rodents) of Pfeiffer, also called the Streptobacillus pseudotuberculosis of Dor.

II. Pseudotuberculoses due to the Bacillus pseudotuberculosis murium (mice) of Kutscher.

III. Pseudotuberculoses due to the Bacillus pseudotuberculosis ovis (sheep) of Preisz.

BACILLUS PSEUDOTUBERCULOSIS RODENTIUM.

Occurrence.—The bacillus causing pseudotuberculosis in rodents is evidently a saprophyte encountered extensively in the outside world. It has been found in garden earth, the sediments from rivers, contaminated by sewage, dust, fodder, milk, etc. It apparently becomes pathogenic occasionally, and then causes pseudotubercular lesions in rabbits, hares, cats, chickens, pigeons, and even in cattle, swine, and other animals.

Pathologic Lesions.—The pathologic lesions which lead, as a rule, to progressive emaciation and finally death, are preferably found

in the liver and spleen. Here numerous whitish round nodules are formed, varying in size from a millet seed to a pea. They are sharply defined from the surrounding tissue, project somewhat above the surface, and contain a caseous centre. Such nodules are also occasionally found in the kidneys. The abdominal lymphatic glands are enlarged, and likewise contain nodules which have a tendency to become confluent. Nodules are also found in the intestinal wall, and particularly in the rabbit the appendix is a favorable seat for them. They also involve the intestinal mucosa where they have their seat in the lymph follicles. The lungs may likewise be affected, and the process, according to Lignière, may lead to purulent pleuritis and peritonitis. The lungs, according to Nocard, are particularly the seat of numerous nodules in pseudotuberculosis of chickens.

Morphology.—The Bacillus pseudotuberculosis rodentium of Pfeiffer is a short, plump rod of small size, generally 1 to 2 micra long, with rounded ends. In older cultures and in the tissues ovoid forms are seen. It has a marked tendency to form shorter or longer chains, hence Dor called it a streptobacillus. It does not show any marked motility in the hanging drop, but Klein claims to have been able to demonstrate one or two flagella with the aid of van Ermengem's silver impregnation method. The organism can be stained with the ordinary watery anilin dyes; it often shows polar or peripheral staining, particularly the ovoid forms. It is Gram negative. It is difficult to demonstrate it in tissues, because it loses the stain so easily, but it may be shown by Klein's method, which consists in first staining for one minute with anilin water gentian violet and then washing for four minutes in iodine iodide of potash solution. The bacillus does not form spores.

Cultural Properties.—The organism grows well on all of the ordinary laboratory media. On *gelatin plates* it forms superficial, yellowish-brown, fairly thick, irregular colonies with serrated edges, 1 to 2 mm. in diameter. There is a nipple-like elevation in the centre of the colony, surrounded by a marmorated periphery. The deeper colonies are more regularly round than the superficial ones. The gelatin is not liquefied, but a cloudy halo due to the formation of fine crystals appears around it after some time. In *gelatin stick cultures* the growth is best at the surface, rather poor along the stick canal, giving the culture a nail-like appearance. *Bouillon* is clouded after eighteen to twenty-four hours, and a pellicle is formed on the surface. Later a dust-like sediment is formed. The alkalinity of the medium becomes increased. There is no indol formation. On agar and *coagulated blood serum* a surface growth with a mother-of-pearl luster is formed. It has been compared, on account of the iridescence, to a thin film of petroleum on water. The addition of glycerin to gelatin or agar favors the growth. On *potatoes* the growth of cultures isolated directly from lesions caused by the bacillus leads to a yellowish-brown, later a brown growth, which has a certain similarity to the

growth of the glanders bacillus on the same medium. The bacillus of pseudotuberculosis grows well in *milk* without changing its reaction or physical properties. The organism is not very resistant to disinfectants and is easily killed by desiccation. It grows both at room and at incubator temperature.

Natural Infection.—This is probably brought about by ingestion. The animals most susceptible to artificial inoculation are the rabbit, guinea-pig, and mouse. Horses, goats, dogs, rats, and cats are not susceptible to the Bacillus pseudotuberculosis rodentium.

BACILLUS PSEUDOTUBERCULOSIS MURIUM.

This organism, obtained by Kutscher from a mouse which had died spontaneously with pseudotuberculous lesions, is characterized by great polymorphism. It forms in artificial cultures large club- and dumb-bell-shaped individuals, which stain unequally, and often closely resemble certain forms of the diphtheria bacillus. It is Gram negative. The bacillus of mouse pseudotuberculosis forms on *agar* delicate, yellowish, serrated colonies, with short lumpy processes. It grows well on *gelatin*, which it does not liquefy. In gelatin stick cultures a whitish growth, which sends out processes into the mass of the culture medium, is formed along the canal. It clouds bouillon in twenty-four to forty-eight hours and forms a pellicle on its surface. It does not grow on *potatoes*. It is pathogenic to mice if administered by inhalation, subcutaneously or intraperitoneally.

Bongert has investigated a mouse epizoötic, also characterized by pseudotuberculous lesions, and has isolated from the latter a small bacillus somewhat resembling the pseudodiphtheria bacillus. He has named this mouse pathogenic bacterium *Corynethrix pseudotuberculosis murium*. The organism grows aërobically and anaërobically best in the incubator. It is 1 to 2 micra long, 0.5 micron thick, and has a tendency, like the diphtheria and pseudodiphtheria bacilli, to form pallisade-like groups.

BACILLUS PSEUDOTUBERCULOSIS OVIS.

Occurrence.—Pseudotuberculosis of sheep, or *ovine caseous lymphadenitis*, is a disease which was first recognized as something different from true tuberculosis by Preisz, whose discovery, however, did not attract much attention at first. Within a few years, however, it was shown that this disease of sheep is by no means uncommon, and it has since been encountered extensively in Australia, New Zealand, Argentina, the United States, and other countries. According to Sivari 10 per cent. of the sheep slaughtered in Buenos Ayres are infected with the disease. It rarely occurs in young animals, and when it does is limited to one or a few lymph glands. In older

wethers, and particularly in older ewes, the fully developed disease with extensive lesions, due to a long chronic course, is observed.

Pathologic Lesions.—The internal organs of animals which have been sick with this disease for a long time show either smaller or larger nodules, inclosed in a fibrous capsule, containing a caseous material, greenish yellow in color, and resembling the contents of the intestinal nodules due to the intestinal parasite Esophagostoma columbianum. The caseous nodules may reach the size of a walnut; they are generally found in the lungs, spleen, and liver, and more rarely in the kidneys. If the lungs are considerably involved the pleuræ are thickened and adherent; the thoracic cavity often contain a pleuritic exudate. In the liver numerous small nodules looking like miliary tubercles are sometimes found instead of the abscesses with caseous material. According to Noergaard and Mohler, who have studied the disease in this country, the principal changes in cases not too advanced are generally confined to the lymph glands; sometimes only a single gland is involved. The glands most commonly affected are, in the order of their frequency: the prescapular, precrural, superficial inguinal, bronchial, mediastinal, sublumbar, deep inguinal, and scrotal, and rarely the suprasternal and mesenteric glands. Microscopic examination shows that the caseous mass is composed of an amorphous material surrounded by more or less degenerated and also intact polynuclear leukocytes. These are again surrounded by fixed connective-tissue cells of the small mononuclear and the endothelial type. Giant cells have never been found. The inflammatory cells are surrounded by fibrous connective tissue which forms the limiting capsule. The pseudotubercle and the true tubercle, according to Grabert, may be distinguished by the following differential characters. While both kinds of tubercles lead to caseation, the pseudotubercle only very rarely shows calcification, but more frequently desiccation with onion-like moulding of the dried layers. If pseudotuberculous material is inoculated into the anterior chamber of the eye, small nodules appear after two to three days, while true tuberculosis leads to nodular formation only after two to three weeks. The pseudotubercle is softer than the genuine one and not grayish white and translucent like the latter, but white or yellowish and perfectly opaque. In old pseudotuberculous nodules, with caseous material young, small tubercles are not seen at the periphery as in true tuberculosis. Pseudotuberculosis ovis is due to a bacillus discovered in 1891 by Preisz and Guinard.

Morphology.—The Bacillus pseudotuberculosis ovis is a very small, slender rod, only slightly thicker than the bacillus of hog erysipelas. It has rounded ends, and the rods are from two to four times as wide, sometimes even longer, and they vary considerably in shape. The ends are sometimes club-shaped, in other rods they are pointed. The bacilli in the discharge from the nodules are often seen in dense groups, both inside and outside of the cells. In caseous material

ovoid forms are seen. The bacilli stain well with the ordinary watery anilin stains; they are Gram positive; they often do not stain uniformly, and then very much resemble the diphtheria bacillus, but are smaller than the latter. The pseudotubercle bacillus of sheep is not motile and does not form spores. Pure cultures can best be obtained from material taken from the outer zone of the caseous material in closed nodules.

Cultural Properties.—The bacillus grows poorly at room, better at incubator, temperature. The first generation always grows poorly, but the development is better in subsequent transplants. The organism is a facultative aërobe. On *agar* small punctate, grayish-white colonies appear after twenty-four hours, and after six to eight days reach their maximum size of 0.5 to 3 mm.; the circumference of the colonies is serrated, their surface dull and umbilicate, with concentric rings arranged around the depressed centre. In generations subsequent to the first few the growth becomes abundant, the colonies become confluent and form a thin, moist, opaque, slightly folded film, which gives rise to threads when touched with the platinum loop. In agar stick cultures small, roundish, grayish-white colonies appear along the stick canal, and the surface becomes covered with a growth similar to that on agar slants. The addition of *glycerin* to the agar appears to be unfavorable. *Bouillon,* during the first six hours of growth, becomes uniformly cloudy, then a granular sediment is formed, and the supernatent fluid becomes clear again; on the surface a dry, grayish-white, broken-up pellicle adhering fairly firmly to the glass tube at the margin is formed. On *coagulated blood serum* small, moist, shiny isolated colonies appear after thirty-six to forty-eight hours, and after a few days form rhizoid processes into the depths of the medium. The colonies on *horse serum* form a white and on *cattle serum* an intensely yellow pigment. On the latter a yellowish cloudy halo is formed around the colonies after some time. On *potatoes* the bacillus forms a dust-like, dirty white film. It grows in *milk* and does not change its reaction or physical conditions. It does not ferment sugar nor does it form phenol or indol. The bacillus of Preisz has its *temperature optimum* at 37° C., and growth ceases at 43° C. The bacillus is not very resistant to heat nor to the ordinary disinfectants.

Inoculation experiments with the bacillus of Preisz have been made by Noergaard and Mohler, and typical lesions were produced in sheep and guinea-pigs. Rabbits are more resistant; chickens and pigeons are not susceptible to inoculations with the organism.

An equine disease known as *Lymphangitis ulcerosa equorum,* or "Lymphangite ulcereuse du Cheval" (French), has been described in the chapter on Glanders under the head of Pseudoglanders. It is now claimed that the bacillus discovered by Nocard in lesions of horses suffering from this disease is identical with the Preisz bacillus of pseudotuberculosis of sheep.

ACID-FAST BACILLI.

When discussing the morphology of the tubercle bacillus it was pointed out that it has peculiar staining properties. While it is difficult to induce the organism to take up watery solutions of basic anilin stains it is equally difficult to decolorize it after the dye has once penetrated into the substance of the bacillus. The reason for this action is that the tubercle bacillus possesses a form of membrane composed of a waxy material. A few other bacilli act toward stains more or less like the tubercle bacillus and are known under the common name of acid-fast bacilli. Several have been found living in the outside world, evidently as *harmless saprophytes.*

Moeller has discovered and described three varieties of such bacilli. One he found on timothy grass (Phleum arvense), another in manure, and a third in plant dust in barns. The three varieties are easily raised on artificial culture media, on which they grow rapidly, forming on the third or fourth day a yellowish to dark orange pigment. The bacilli are even more firmly acid-proof than the tubercle bacilli, and can still better withstand dipping in dilute mineral acids and washing in alcohol. The first two varieties of Moeller's acid-fast bacilli grow best at temperatures of 45° to 50° C.; when obtained from young cultures they show some motility in the hanging drop, while older cultures display considerable pleomorphism with branching forms.

Moeller's grass bacillus when inoculated intraperitoneally into guinea-pigs leads to pseudotubercular lesions and the formation of abscesses containing a purulent or somewhat caseous material. Typical tubercles with giant and epithelioid cells are, however, not formed. Moeller also obtained an acid-fast bacillus from a nodule of a steer; it grew very rapidly at room temperature, and in intraperitoneal inoculation produced a pseudotuberculosis in guinea-pigs.

Petri and Rabinowitsch have isolated from butter, acid-fast bacilli which closely resemble the acid-fast bacilli of Moeller. Petri found such bacilli 54 times in 102 specimens of butter examined; Rabinowitsch 23 times in 80 specimens examined; Klein in London in 8 out of 100 specimens, and Santori in Rome in all specimens of butter tested for the presence of such organisms. The Petri-Rabinowitsch bacilli are not as firmly acid-fast as the tubercle bacillus. When raised on agar they form a thick, cream-like growth, which later assumes an orange color, then shrivels and becomes cracked and uneven. If repeatedly passed through animals the growth becomes dry and cracked, and closely resembles a culture of tubercle bacilli on glycerin agar. Bouillon cultures of the Petri-Rabinowitsch acid-fast bacilli remain clear and become covered with a thick folded membrane which gives off a disagreeable ammoniacal odor and forms a small amount of indol. If these butter bacilli are inoculated intraperitoneally into guinea-pigs they produce pseudotubercular lesions.

It has been shown by experiments made by investigators upon themselves that these butter bacilli are not pathogenic to man. Korn isolated in butter in Freiburg a bacillus now generally known as the Bacillus Freiburgensis. It has nearly the same staining and cultural properties as the other butter bacilli, and is pathogenic for white mice but not for guinea-pigs.

SMEGMA BACILLUS.

There occurs on the human skin, particularly in the smegma under the prepuce and between the folds of the labia majora and minora of the female, an acid-fast bacillus which, in connection with human excretions, such as urine, etc., may be confounded with the tubercle bacillus. This acid-fast bacillus is known as the smegma bacillus. According to Fränkel and Neufeld there are two varieties: one, called the "tuberculoid," is more slender and stains a bright scarlet red; the other, known as the "diphtheroid," stains more purplish red, and is easier to decolorize than the former. According to Fränkel the latter variety only has been grown in artificial cultures. It develops much more rapidly than the tubercle bacillus, and on most of the ordinary media. When these bacilli are cultivated for several generations on artificial media they lose their acid-fast character, and can be easily decolorized.

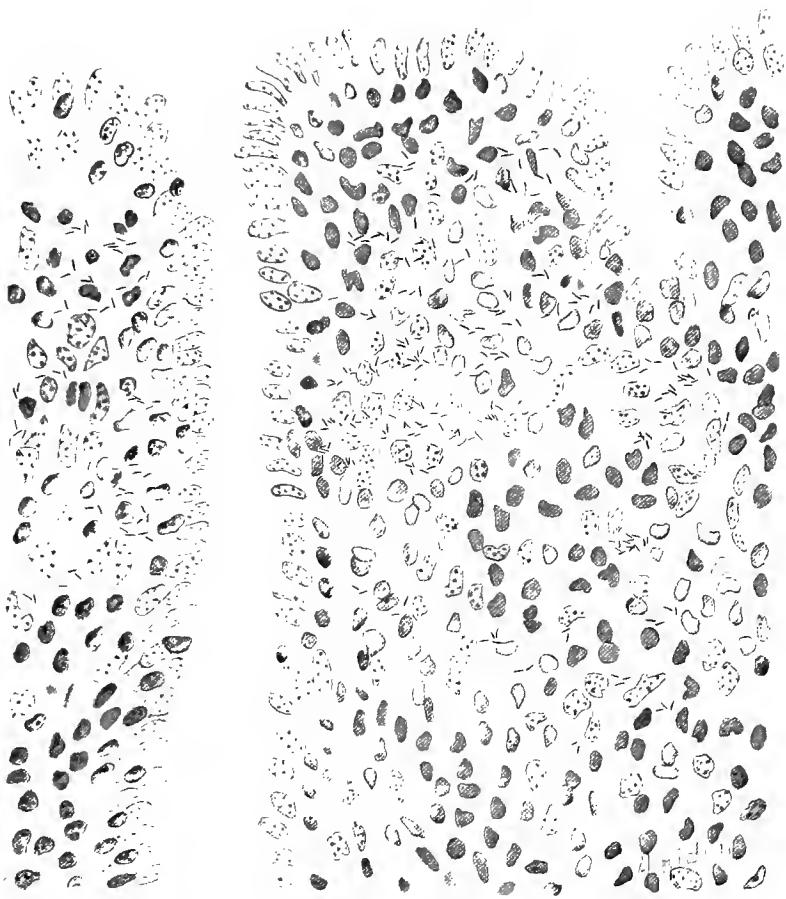
THE BACILLUS OF LEPRA IN MAN AND RATS.

The human disease *leprosy*, or *lepra*, is very probably due to an acid-fast bacillus which occurs in enormous numbers in the pathologic lesions of this disease. The bacillus has never been successfully obtained in pure cultures, but certain methods like those of Weil and Clegg bring about a multiplication of lepra bacilli which, however, are not present in pure cultures. The author has seen lepra bacilli multiply by obtaining material from lepra tubercles, inclosing it in collodion sacs and implanting these into the peritoneal cavities of monkeys, where they were left for several weeks. The material became completely softened under these conditions and showed numerous lepra bacilli, which, when inoculated into other monkeys, however, failed to produce the typical picture of the disease. Several French authors, however, have claimed that they were able to infect monkeys with human leprosy.

The bacillus of human leprosy is easier to stain but not as firmly acid-proof as the tubercle bacillus, and it can, therefore, be distinguished from the latter by the following method recommended by Baumgarten:

To a watch-glassful of distilled water add 5 to 6 drops of a saturated alcoholic solution of fuchsin; allow the cover-glass with the leprous or tuberculous material on it to float on the surface of the stain for six or seven minutes. Decolorize one-quarter of a minute in 10 per

PLATE XI



Section of the Small Intestine of a Cow Dead from Chronic Bacterial Dysentery. (Johne's Disease.)

Oil immersion magnification.

cent. nitric acid alcohol, then wash in distilled water and counterstain with watery methylene blue. This method stains the lepra bacillus red, while tubercle bacilli are not stained at all.

Rat Leprosy.—There is a disease in wild gray rats which is caused by an acid-fast bacillus and which presents lesions almost identical with those found in human leprosy. The two diseases, however, are not identical. The rat cannot be infected with human leprosy, and there is no reason to believe that the human disease came originally from rats or that man can be infected with rat leprosy.

Occurrence.—Rat leprosy has been observed by Stefansky in Russia, Rabinowitsch in Berlin, Dean in London, Tidswell in Australia, the English Plague Commission in India, and Wherry and McCoy in California; accordingly, it appears to occur over a large territory. Its prevalence bears no relation to human leprosy, as is shown in the Hawaiian Islands, where leprosy among human beings is common and where, in spite of rewards offered, leprosy-infected rats have never been found.

Pathology.—The pathologic lesions of this disease of rats, according to Brinckerhoff, are as follows: The skin in a well-developed case of the disease presents a patchy alopecia coincident with thickening and nodule formation situated in the subcutaneous tissue. The cut surface of the nodules or thickenings is light yellow in color, clean, dry, and cheese-like. In the region of the nodules the skin is atrophic, and ulcers often form on the prominent parts of the affected area. The subcutaneous fatty tissue is diminished in amount. Histologically the process is seen to be practically confined to the subcutaneous tissue and to consist essentially in the presence of cells rich in protoplasm, with vascular nuclei, whose cell body is more or less completely filled with slender acid-fast bacilli. The subcutaneous fat is replaced by such a tissue. Where the musculature is involved the muscle fibers atrophy and the fibers are infiltrated with the acid-fast bacilli. The peripheral lymph nodes are generally involved; they are enlarged and on section opaque, pale yellowish-white in color.

Morphology of Bacillus of Rat Leprosy.—It resembles closely the lepra bacillus of man, is 3 to 5 micra long, 0.5 micron wide; it is somewhat more firmly acid-proof than the human lepra bacillus. It often shows a beaded appearance, and presents itself in crowded bundles. The disease can be transmitted by inoculation to gray and white rats. Guinea-pigs and other animals are not susceptible.

BACILLLUS OF CHRONIC DYSENTERY IN CATTLE (JOHNE'S DISEASE).

Occurrence.—There occurs in cattle a chronic dysentery with progressive emaciation and anemia and finally death, due, as it appears, to an acid-fast bacillus which is found in enormous numbers

in the dysenteric discharges and in the mucosa particularly of the small intestines. The disease was first recognized as one of a peculiar type by Johne and Frothingham in 1895. They found the acid-fast bacillus, and were at first inclined to look upon it as an avian tubercle bacillus. Other cases were then reported in Europe by Markus, Lienan and Van den Eeckhout, Borgeaut, and Bang. The first cases in the United States were noticed by Pearson in Pennsylvania and by Beebe in Minnesota. Since then not a few cases have been observed in this country. The author received material from a case in 1908 through the kindness of Professor Alexander, of Madison, Wis., and a second case likewise from Wisconsin, through Dr. Hermann Schwarze.

FIG. 153

The small intestine of a cow in chronic bacillary dysentery. (Johne's disease.)

Pathologic Lesions.—Animals dead from chronic bacterial dysentery upon postmortem examination are generally found to be very much emaciated and extremely anemic. There are otherwise no marked changes in the internal organs except in the intestines, which are the seat of characteristic lesions. The small intestine in particular shows great thickening. If, however, the disease has been recognized early by microscopic examination and inoculations and the animal has been slaughtered while still in good condition the thickening may be moderate; but if death has occurred both the mucosa and the submucosa of the small intestine are much thickened. The former develops coarse, very prominent corrugations, which show a congested and sometimes even hemorrhagic surface. These changes may extend from the small to the large intestines. Microscopic examination of sections of the mucosa of the small intestine shows that the thickening is due chiefly to the increase in fixed connective-tissue cells and to infiltration by an edematous exudate. There are neither true tubercles nor giant cells, but between the cells enormous numbers of acid-fast bacilli are encountered. These are much more numerous than in any form of tuberculosis, including avian tuberculosis. There is a striking similarity in morphology, numbers, and grouping between the acid-fast bacilli of this bovine chronic dysentery and the bacilli of human lepra.

Morphology.—The bacillus of chronic dysentery of cattle is a rod 2 to 3 micra long and 0.5 micron wide. It presents itself in regular

cylindrical, in slightly curved, and in club-shaped forms. It has the same staining properties as the tubercle bacillus, is acid-fast, and apparently can well withstand long washing in alcohol.

Attempts to cultivate the bacillus, as well as animal inoculations, have so far been unsuccessful. Since the disease always appears to lead to a fatal issue, animals found to be infected with it should be slaughtered as soon as the diagnosis is made. The differential diagnosis from intestinal tuberculosis can be made by the tuberculin test and by inoculation experiments upon guinea-pigs.

QUESTIONS.

1. What is meant by pseudotubercular diseases?
2. Name the three types of bacilli which cause such pseudotubercular diseases.
3. Are these bacilli acid-fast? What is the meaning of the term acid-fast as applied to bacteria?
4. What is the Bacillus pseudotuberculosis rodentium?
5. Is it found as a saprophyte, and if so, where?
6. What animals are susceptible to the natural infection by this bacillus? By what route does it generally enter? What animals are susceptible to artificial infection?
7. Describe the lesions produced by the Bacillus pseudotuberculosis rodentium
8. Describe its morphology and staining properties.
9. Describe its cultural properties.
10. What is the Bacillus pseudotuberculosis murium?
11. Describe its morphology and cultural properties and the lesions which it produces.
12. What kind of disease is ovine caseous lymphadenitis? Where does it occur?
13. Describe the pathologic lesions of the disease.
14. What causes the disease? Who discovered its specific microörganism?
15. Describe its cultural characteristics.
16. What animals are susceptible to the disease (*a*) under natural conditions; (*b*) when inoculated artificially?
17. What is the relation between the bacillus of Preisz (in ovine pseudotuberculosis) and that of Nocard in lymphangitis ulcerosa equorum?
18. What are the three acid-fast bacilli of Moeller?
19. What effect have these bacilli when inoculated intraperitoneally into guinea-pigs?
20. What are the Petri and the Rabinowitsch butter bacilli?
21. Describe their growth on agar.
22. Describe their effect in intraperitoneal inoculation into guinea-pigs.
23. How can the characteristics of their growth on agar be changed so that cultures resemble those of the tubercle bacillus?
24. What is the smegma bacillus?
25. How can the human lepra bacillus be distinguished from the human or bovine tubercle bacilli?
26. Is lepra in man and lepra in the rat identical?
27. Describe the pathologic lesions in leprosy of rats.
28. Describe the bacillus of rat leprosy.
29. Can this disease be transmitted artificially, and to what animals?
30. Where has rat leprosy been observed?
31. What kind of disease is chronic bacillary dysentery of cattle? By what other name is it also known?
32. Describe its bacillus and the pathologic lesions produced by it.
33. Name four different diseases in animals due to different acid-fast bacilli. How can you distinguish these four species by stains, cultural and inoculation tests?

CHAPTER XXXII.

VARIOUS COCCI PATHOGENIC FOR DOMESTIC ANIMALS AND MAN —DIPLOCOCCUS MENINGITIDIS EQUI—DIPLOCOCCUS INTRACELLULARIS—DIPLOCOCCUS PNEUMONIÆ—MICROCOCCUS CATARRHALIS—PNEUMOCOCCUS OF FRIEDLÄNDER —GONOCOCCUS—MICROCOCCUS CAPRINUS— MICROCOCCUS MELITENSIS.

INFECTIOUS EQUINE CEREBROSPINAL MENINGITIS.

Historical and Occurrence.—Epizoötic cerebrospinal meningitis among horses has evidently been observed for a long time. It was described in 1813, in Germany, by Woertz, as "Hitzige Kopfkrankheit" (febrile disease of the head). A more extensive epizoötic in Europe was described by Franqué in 1824. Large (1847) and Liautard first described epizoötics in the United States, while more recent contributions have come from Wilson and Brimhall and Streit and Harrison. The disease was very prevalent in 1878 and 1879 in Saxony, particularly in the neighborhood of Borna, and it has consequently been occasionally designated as *Borna disease.*

Pathologic Lesions.—The anatomic changes in the disease, according to Hutyra and Marek, are generally not well marked to the naked eye. They consist in dilatation of the vessels of the pia-arachnoid of the cerebrum and cord and the presence of a clear yellowish, not purulent, exudate in the ventricles. Moore, in the examination of a number of cases, found no marked lesions visible to the naked eye. MacCallum and Buckley have found foci of softening in the frontal areas anterior to the motor region of the cortex. It appears, however, that the cases examined by McCarthy and Ravenel were not of the true epidemic, but of another type. Sometimes a fibropurulent exudate has been found at the base of the brain and at the medulla oblongata.

Bacteriology.—A number of observers have studied the bacteriology of the disease. Siedamgrotzky and Schlegel have found a coccus which generally presents itself singly and more rarely as a diplococcus. It is Gram positive. On gelatin plates it forms dirty, grayish-white, well-defined colonies, with a denser centre; on agar, white, shining, sharply defined colonies. The organism clouds bouillon. Johne found a coccus which, while quite similar to the one just described, varied from it in some details, and was often seen in short chains and intracellularly in leukocytes. It was, therefore, much like the Diplococcus intracellularis of Jaeger-Weichselbaum, which

causes epidemic cerebrospinal meningitis in man. Ostertag found cocci very much like those of Johne; they are Gram negative and closely resemble the organism causing the human disease. The organism of Ostertag, when inoculated subdurally into horses, produced a more or less typical attack of cerebrospinal meningitis. Wilson and Brimhall, in cases of cerebrospinal meningitis in horses and other domestic animals, found both cocci of the type of the Jaeger-Weichselbaum organism and of Fränkel's pneumococcus. It appears, therefore, that cerebrospinal meningitis in the horse, like cerebrospinal meningitis in man, may be caused by more than one bacterium; the etiology, however, of the disease in equines is not yet satisfactorily cleared up and further investigations are necessary.

Cerebrospinal meningitis has also been occasionally found in cattle, sheep, and swine. The description of the disease in these animals is still very fragmentary.

Johne has named his organism *Diplococcus intracellularis equi.*

DIPLOCOCCUS INTRACELLULARIS MENINGITIDIS.

This organism, first seen by Weichselbaum and later carefully studied by Jaeger, is generally the cause of cerebrospinal meningitis in man. It is a biscuit-shaped diplococcus, and frequently occurs in large numbers in the protoplasm of pus corpuscles of the fibrino-purulent exudate found at the convexity and at the base of the brain in human cerebrospinal meningitis. The diplococcus stains well with the ordinary watery anilin stains, but is easily decolorized when treated by Gram's method. Pure cultures can generally be obtained from cerebrospinal fluid, drawn with aseptic precautions. Of this fluid 1 to 2 c.c. is inoculated into blood serum or glycerin-agar tubes. Pus in cerebrospinal meningitis can be obtained by spinal puncture. This consists in the introduction of a strong hypodermic needle, 4 to 8 cm. long, attached to an all-glass (Luer) large hypodermic syringe, into the spinal canal, about 1 cm. from the middle line and between the third and fourth lumbar vertebræ. The diplococci of cerebrospinal meningitis grow in the incubator at blood temperature only. They develop around surface colonies with an opaque yellowish-brown centre having a flat, thin periphery; the edges may be circular and straight or dentate. Subcutaneous inoculations into laboratory animals are generally not pathogenic for them, but intraperitoneal and intravenous injections often cause a fatal result in mice and rabbits. Flexner and Joblin have prepared an antimeningitis serum in the horse which has proved an excellent therapeutic agent in the human disease, but it must be injected into the spinal canal in comparatively large doses (15 to 30 c.c.) often repeatedly, and it is necessary each time first to withdraw an equivalent or larger amount of the purulent cerebrospinal fluid.

DIPLOCOCCUS PNEUMONIÆ OF FRÄNKEL-WEICHSELBAUM.

In a great majority of cases this organism is the cause of croupous or lobar pneumonia in man; it also causes inflammatory and suppurative processes in the middle ear, the joints, the pleura, the peri- and endocardium, and sometimes cerebrospinal meningitis. It is also called *Diplococcus lanceolatus*, and was probably first seen by Sternberg and Pasteur. The typical shape of the organism, when seen singly and in pairs, is the elongated coccus. The diplococci show particularly often in the shape of candle lights, hence the name *Diplococcus lanceolatus*, which means lancet-shaped. In pathologic exudates the organism frequently appears in short chains in which the subdivision into diplococci is generally well marked, and it almost invariably shows a distinct, quite large capsule, because of which it is known also as the *capsule coccus of pneumonia*. As a rule, the capsule is not shown on artificial culture media, but sometimes it appears on sterile sputum or in sterile fluid blood serum or milk. The diplococcus of pneumonia does not form spores, is not motile, and possesses no flagella. It is Gram positive. The organism, after a number of transplants, often forms longer chains, and the individual cocci become perfectly round and lose the lancet-shape entirely. It grows rather poorly on artificial culture media and only at blood temperature, both aërobically and anaërobically. The superficial cultures on *agar* are thin films, quite transparent. The deeper colonies are very small, and when examined with a low power of the microscope appear light yellow or light brown, and finely granular. The growth on *gelatin plates* kept at 24° C. is scanty and poor. In *agar stick cultures* there is very little growth on the surface, but more along the stick where a band-like strip is formed. On *agar* or *blood-serum slants* a fine veil composed of small dewdrop-like colonies is formed. Nutrient *bouillon* and *fluid blood serum* become cloudy, and a scanty, loose, whitish sediment is formed at the bottom. The growth on *potatoes* is practically invisible, and is only revealed by microscopic examination of material taken from the surface. *Milk* becomes coagulated. The addition of *glycerin* and *glucose* to the media is favorable to the growth. The reaction of the culture soils must be faintly alkaline. The best medium is a *serum agar* prepared of two parts of agar to which one part of human-blood serum has been added. The organism generally dies out rapidly in artificial cultures. Sometimes a second generation fails. It is always necessary to make frequent transplants. The coccus perishes so quickly in pure cultures because it rapidly forms acid in the presence of which it cannot live. The virulency shown by different stems in first cultures varies greatly; it is lost rapidly in subsequent transplants. It appears from Rosenou's work that virulent diplococci are unaffected by phagocytosis, avirulent types alone are engulfed and destroyed by leukocytes. While pneumonia

in man is in a majority of cases caused by the Diplococcus lanceolatus, it may also be caused by the *Diplococcus* or *Diplobacillus of Friedländer*, the *Micrococcus catarrhalis*, and the *bacilli of influenza, glanders, diphtheria, plague*, and *others*.

Whether every case of contagious pneumonia of horses is always due to equine influenza, *i. e.*, to the Bacillus bipolaris equisepticus,[1] or whether certain non-contagious pneumonias in horses are due to other bacteria is a question on which opinion is divided. It, however, appears established that the diplococcus pneumoniæ or lanceolatus is also the cause of pneumonia in the horse, and that in old animals, or after long-continued debilitating diseases it frequently leads to a fatal issue.

MICROCOCCUS CATARRHALIS.

This was first seen by Seiffert and obtained in pure cultures by Kirchner and by Pfeiffer. It is a biscuit-shaped coccus, generally occurring in pairs or tetrads. It stains well with the ordinary watery anilin stains, but is Gram negative. It is not motile, and does not form spores. It is found in the normal mucous membranes, and also in diseases of the upper or lower respiratory tract in man. It grows on the ordinary laboratory culture media between 20° and 40° C., best at blood temperature. It forms grayish-white or yellowish-white colonies with irregular margins, as if artificially cut out. *Gelatin* is not liquefied; *bouillon* becomes cloudy, a pellicle often forming on the surface. *Milk* is not coagulated. The organism varies considerably in virulency. It sometimes kills guinea-pigs, rabbits, and mice in intraperitoneal inoculations.

PNEUMOCOCCUS OF FRIEDLÄNDER.

This organism should more properly be called the *Pneumobacillus of Friedländer*, as it is not a true coccus but a short bacillus. In pathogenic exudates it is often so short that it was at first mistaken for a coccus. It generally occurs in groups of two or in tetrad form or in short chains, and when found in sputum or pus frequently possesses a capsule. It can easily be differentiated from the Diplococcus pneumoniæ of Fränkel because it is Gram negative. It does not form spores, is not motile, and possesses no flagella. It is aërobic. It shows a nail growth in *gelatin stick culture, i. e.*, it forms a heavy growth on the surface from which a streak of culture extends along the stab. It does not liquefy the gelatin, but imparts to it a distinct brownish tint distinguishing it from the very similar growth which the Bacillus aërogenes forms on this medium. The growth on

[1] Described in the chapter on the Hemorrhagic Septicemia Bacilli.

gelatin plates at the end of twenty-four hours shows small, white, circular colonies, which increase rapidly in size. The colonies on *agar* are grayish white and moist. On *blood serum slants* an abundant grayish-white, moist, and shiny growth is formed, and on *potatoes* a thick, yellowish-white shining layer. *Milk* is not coagulated. Lactose and glucose are fermented with the formation of acid. The diplobacillus of pneumonia has not been encountered as the natural cause of disease in domestic animals.

GONOCOCCUS.

The gonococcus, or Micrococcus gonorrhœæ, is an organism occurring in pairs and occasionally in tetrads. The individual cocci forming the pair are flattened at their opposing surfaces, often somewhat resembling a kidney or coffee bean in form. The organism is the cause of gonorrheal inflammation of the urethra in men and women. It also penetrates deeper into the genito-urinary tract, and causes epididymitis, ovarian abscess, salpingitis, inflammation of the pelves of the kidneys (pyelonephritis), peritonitis, inflammation of the joints, and endocarditis. It is generally spread in sexual intercourse. It may also be transferred with the fingers or otherwise to the eye and cause a very dangerous conjunctivitis. The organism in pus is frequently found intracellularly in the protoplasm of the leukocytes. It stains very rapidly with the watery anilin stains, but also loses the stains very easily, and is Gram negative. It is difficult to grow on artificial culture media. The best medium is an ordinary agar, two parts, to which one part of sterile human-blood serum[1] has been added. The ingredients are mixed after the melting of the agar and again solidified after inoculation from fresh gonorrheal pus. Gonococci form colonies on such *serum agar* after sixteen to twenty-hours, but they are so small as to be only visible with a good hand magnifying glass or the low power of the microscope. After twenty-four hours the colonies have attained the size of a pinhead and do not grow much larger. They are light gray, translucent, and of a peculiar tenacious, slimy consistency. They generally remain round, well defined, and do not, as a rule, become confluent, but may touch each other. The culture soil in transmitted light somewhat resembles the surface of ice which has been much cracked. In exceptional cases the colonies become confluent and form grayish-white films. Colonies of gonococci closely resemble colonies of the Diplococcus lanceolatus, but the former are generally somewhat larger, less translucent, and more highly colored. Gonorrhea is a strictly human disease. It does not occur naturally among animals, and even artificial inoculation is very difficult and rarely successful. Mice in intraperitoneal infection develop a localized peritonitis.

[1] Ascitic, hydrocele, or pleuritic fluid may be used in place of the human-blood serum in the preparation of the medium.

MICROCOCCUS CAPRINUS.

Under this name Mohler and Washburn have described a coccus which is claimed to be the cause of *takosis* (*i. e.*, a wasting disease of Angora goats). The most characteristic morbid lesions of this affection are emaciation and anemia. Consolidated areas are generally found in the lungs, the myocardium is pale, soft, and flabby, and the epicardium and endocardium may present hemorrhagic spots. The kidneys are anemic and softened and the spleen is small and may be adherent to the neighboring structures. The Micrococcus caprinus has been isolated from the heart's blood. It usually presents itself in pairs, and is pathogenic for goats, chickens, rabbits, guinea-pigs, and white mice, but not for sheep, dogs, or rats.

MALTA FEVER AND THE MICROCOCCUS MELITENSIS.

Occurrence and Historical.—Malta or Mediterranean fever is a septicemic disease of man, caused by the Micrococcus melitensis, discovered by Bruce in 1887. It is, however, by no means confined to Malta, as cases have been reported from Greece, Italy, Spain, Turkey, North and South Africa, America, India, and the Philippine Islands. The affection is of particular interest to the veterinarian because it is primarily a disease of goats and is transferred through their raw milk to man.

Morphology.—The Micrococcus melitensis is a very small coccus, measuring about 0.4 by 0.3 micron. It is seen as a monococcus or as diplococci, more rarely in short chains. The diameter of the individual stained cocci, according to Bruce, is about 0.33 micron. Longer chains of 10 to 14 cocci are frequently seen in older bouillon cultures. The union of the individual links is evidently not very firm and the chains in stained preparations are broken up into smaller segments. Involution forms, which come to resemble bacilli, are found in older cultures. The organism does not form spores, is not motile, and has no flagella. It stains well with the ordinary anilin dyes, and is Gram negative.

Cultural Properties.—The organism grows best in culture media which are slightly acid to phenolphthalein + 1 (alkaline to litmus). It grows best at blood temperature, but has a wide range of temperature. The growth is slow, and it generally requires three to four days before colonies can be seen with the naked eye. The surface of *agar plates* liberally inoculated after twenty-four to thirty-six hours somewhat resembles ground glass under a low power of the microscope. After twenty-four to seventy-two hours individual dewdrop-like colonies are visible. In about eight days they reach a diameter of 1.5 mm. They are round, regularly spherical; the superficial colonies

are convex, with a darker centre. The deep colonies are biconvex, with a smooth margin. According to Eyre the best culture medium is *litmus nutrose agar,* prepared with cattle serum. The reaction on this medium is not changed and the litmus preserves its bluish color. In *fluid serum* the coccus forms a fine flocculent precipitate; the supernatant fluid generally remains clear; a clouding occurs only rarely. The growth on *gelatin* is quite slow. Liquefaction does not occur. *Nutrient bouillon* after seventy-two hours becomes uniformly cloudy, but after eight days a sediment begins to form, and after four weeks the entire growth has sunk to the bottom and the supernatant fluid is again clear. The growth on *potatoes* is very scanty. Sugars are not fermented. The organism is easily destroyed by moist heat, less easily by dry heat. It can withstand drying out for a considerable time, but is easily killed by direct sunlight, less easily by diffuse daylight. The organism is pathogenic in artificial inoculation to monkeys, rodents, equine, cattle, sheep, and goats. The Mediterranean Fever Commission found the organism in the milk of goats on Malta, and the blood of such animals exhibited specific agglutinative power for the coccus. Goats can be infected by feeding and subcutaneous and intravenous injections, and the organism can afterward be recovered in the milk. Anderson, 1905, reports an outbreak of Malta fever in a crew of a vessel who had been consuming milk from goats bought in Malta for importation into the United States. Examination of some of the goat's milk showed infection with the Micrococcus melitensis.

QUESTIONS.

1. Where has epidemic or infectious equine cerebrospinal meningitis been observed?
2. What organisms have been held to be the etiologic factor in this disease?
3. Describe the pathologic lesions of the disease.
4. What are the views as to the relation of the Diplococcus intracellularis equi and the Diplococcus intracellularis of Weichselbaum?
5. How can the latter be obtained in pure culture?
6. Describe its morphology and cultural characteristics.
7. To what organism is human pneumonia generally due?
8. Describe its morphology and cultural properties.
9. In what other human diseases has it been found?
10. What is most commonly the cause of pneumonia in the horse?
11. Does the Diplococcus lanceolatus ever cause pneumonia in the horse?
12. Describe the Micrococcus catarrhalis. Where found? Does it cause disease?
13. Describe the pneumococcus of Friedländer. Is it Gram + or —?
14. Describe the gonococcus. What animal diseases does it cause?
15. How can it be obtained in pure cultures?
16. What is takosis in goats? What organism causes it?
17. What is Malta or Mediterranean fever?
18. What organism causes it?
19. Describe its morphologic and cultural properties.
20. How contracted by man?

CHAPTER XXXIII.

SPIRILLA—PATHOGENIC VIBRIONES—SPIROCHETE—THE VIBRIONES OF CHICKEN SEPTICEMIA AND OF ASIATIC CHOLERA—SPIROCHETE IN MAN, OTHER MAMMALS AND BIRDS.

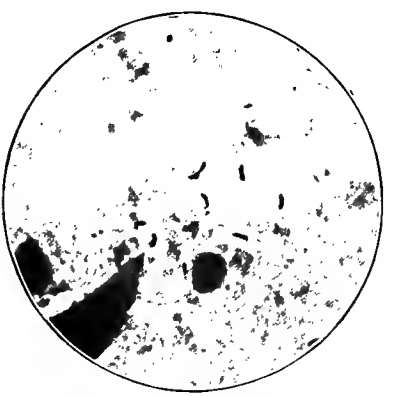
The Systematic Classification of Spiral Bacteria.—The spiral bacteria may be divided into two groups. One comprises curved, rod-like organisms shaped very much like a comma. They form a real spiral only when a number adhere together. The other group is composed of organisms which are true spirals, their bodies representing wavy filaments which may best be likened to corkscrews or winding stairways. Members of the first group are designated as *Vibrio* (pl. vibriones); members of the second as *spirillum* (pl. spirilla) or *spirocheta* (pl. spirochete). The term spirillum, however, is not used in a very strict manner and the comma-shaped organism of Asiatic cholera is known both as the vibrio and the spirillum of Asiatic cholera, while in accord with strict nomenclature it should be known as a vibrio exclusively. The organisms of the type of vibrio or spirillum are generally very lively motile. The motility of vibriones does not differ much from that of lively motile bacilli, but the motion of the spirochete is peculiar and characteristic. It consists in a rotation around their long axis, forward and backward like a spiral spring released from compression, and a bending motion of the whole body. The vibriones generally possess a single flagellum at one end, but flagella at both ends or multiple flagella at one or both ends are also encountered. Spirilla may have one flagellum at one end, one at either end, several flagella at one end, or flagella surrounding the entire body. There has never been any doubt in regard to the classification of the vibriones among the bacteria, *i. e.*, among the vegetable microörganisms. Recently however, it has been claimed, particularly by Schaudin and others, that the spirochete are protozoa and near relatives of the trypanosomes, if not indeed themselves true flagellata of this type. The question will be considered fully at the end of this chapter. Most vibriones are harmless saprophytes, but two species at least possess very pathogenic properties.

VIBRIONES.

Vibrio Metchnikovi, or Spirillum of Metchnikoff.—In the investigation in 1887 of an epizoötic among chickens in Odessa, Gamaleia dis-

covered as its cause a comma-shaped bacterium which he named in honor of Metchnikoff. The disease is generally fatal after forty-eight hours, and its characterized by violent diarrhea without fever. Postmortem examination shows the intestinal mucosa inflamed, its epithelium desquamated; the contents of the intestines are fluid and mixed with blood. The fluid contents contain enormous numbers of comma-shaped bacteria, which very much resemble the spirillum of Asiatic cholera in man (see below). For this reason the disease has been called vibrio cholera or vibrio septicemia of chickens. The spirillum of Metchnikoff, as found in the intestines of dead chickens, occurs, in addition to the typical comma shape, also in shorter, thicker specimens, which look almost like cocci. The vibrio is lively, motile, and possesses one long slender flagellum. The organism forms longer spirals in older cultures in which the individual commas adhere to each other in chains. The spirillum of Metchnikoff stains well with watery fuchsin, and is Gram negative. It grows well in the ordinary laboratory media, and the cultures closely resemble those of the spirillum of Asiatic cholera. The vibrio of chicken septicemia grows rapidly on *gelatin* plates at room temperature, and after twenty-four to thirty hours has formed pinhead-sized circular colonies, which rapidly liquefy the medium. The colonies under a low power of the microscope appear granular and yellowish or brownish. Older cultures which have been transplanted for a long time on artificial media do not liquefy gelatin as rapidly as cultures recently isolated from infected animals. The growth in *gelatin stick cultures* is much more rapid than that of the vibrio of Asiatic cholera. *Bouillon* is rapidly clouded and a fairly strong white pellicle forms on the surface of the medium. On *agar* the vibrio forms a yellowish, on *potatoes* a yellowish-brown, growth. The vibrio of Metchnikoff cannot be safely distinguished by any of its cultural characteristics from the vibrio of Asiatic cholera. Both also form indol and nitrites in bouillon, and these upon the addition of a few drops of chemically pure sulphuric acid give a red color reaction. The differentiation between the two organisms, however, can be made by animal experiments. The vibrio of Metchnikoff kills pigeons if injected into the thoracic muscles within twenty-four hours. Postmortem examination of the dead birds shows the injected muscle swollen, yellowish in color, and infiltrated,

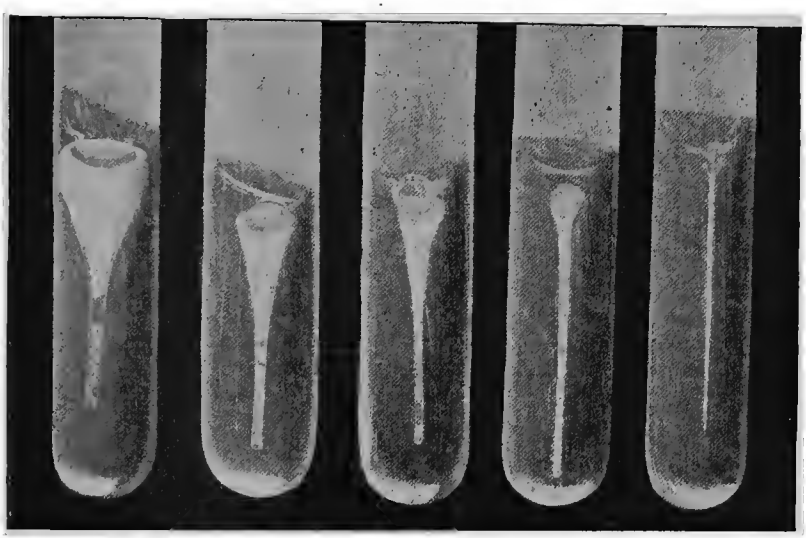
FIG. 154



Spirillum of Asiatic cholera smear from the intestines of a man dead from cholera. × 1000. (Author's preparation.)

with a serous fluid which contains innumerable spirilla. The blood also contains many spirilla. The mucosa of the intestines is pale and the intestinal contents are liquid and likewise contain the organism. Guinea-pigs also generally succumb to subcutaneous infection within twenty-four hours. A hemorrhagic edema is formed at the place of inoculation, and the fluid and blood show enormous numbers of vibriones. The vibrio of Asiatic cholera, on the contrary, in subcutaneous or intravascular injection never produces a rapidly fatal septicemia in these animals, which are so susceptible to the spirillum of Metchnikoff. In spite of their great morphologic and cultural similarities the two organisms are by no means identical. They are very different in their pathogenicity and animals cannot be immunized with one species against the other.

FIG. 155



A characteristic series of cholera cultures in gelatin; from right to left, one, two, three, four, and six days' growth. (Dunham.)

Vibrio of Asiatic Cholera.—This organism was discovered by Robert Koch in India. Asiatic cholera is a disease of man and does not naturally occur among the lower animals. The disease is endemic in India, but has spread from there on various occasions into Europe, America, and other countries. It is one of the most fatal epidemics of mankind, and has a very high mortality. The great similarity between the spirillum of Asiatic cholera and the Vibrio Metchnikovi has already been referred to above; it also closely resembles several other spirilla, which will be described later.

The cholera vibrio, also called the comma bacillus of Koch, is a curved organism, and in artificial cultures often forms longer spirals

composed of individual commas adhering together. It is actively motile, possesses one flagellum, and has, like all the organisms of this group, the same staining properties as the vibrio of Metchnikoff. In older cultures very irregular involution forms are seen. Pure cultures of the cholera spirillum can best be obtained by mixing fecal matter containing it with *nutrient bouillon* in a flask. When kept at blood temperature in the incubator a white pellicle forms on the surface. This is chiefly composed of rapidly growing vibriones, and if tubes are inoculated from the pellicle, and *gelatin plates* poured, pure cultures can be obtained. In the lower stratum of the gelatin plates small white dots appear, which grow up to the surface and liquefy the medium. The color of the colonies soon turns yellowish. In *gelatin stick cultures* the liquefaction leads to a funnel-shaped excavation of the surface. The organism grows in *milk*, which it does not visibly change, but under natural conditions it is soon killed in milk because it is very sensitive to acid.

Vibrio Proteus,[1] or the Spirillum of Finkler and Prior.—This organism was discovered by Finkler and Prior in the feces of persons sick with diarrhea. The spirilla are curved and somewhat longer and coarser than the cholera vibrio; they are often pointed at the ends and thicker in the middle. In artificial cultures they form spirals which generally are not as long as those of the two preceding vibriones. When the culture medium is not very favorable the spirilla vary greatly in shape, and often form large oval bodies or very coarse curved bacilli. For this reason the organism has been called *Vibrio proteus* by Buchner. The colonies on *gelatin* are darker and more regularly circular than those of the cholera vibrio. The liquefaction in *gelatin stick cultures* is very energetic, and progresses to a sacculate zone within forty-eight hours. The organism ferments sugar with the formation of acid and produces a fetid smell in all culture media. The spirillum of Finkler and Prior is probably not pathogenic to man, but only a harmless saprophyte occasionally found in the intestinal tract. It is very slightly pathogenic to animals in subcutaneous and intraperitoneal injection.

Spirillum of Denecke, or Vibrio Tyrogenum.—This organism belongs to this group. It is not pathogenic, and was first found in old cheese by the author whose name it bears. It is somewhat smaller than the vibrio of cholera, and in artificial cultures forms long, slender spirilla.

Water Spirilla.—A number of spirilla of this group have been found in the water of rivers in Europe and America, such as the Vibrio Berolinensis (found in Berlin), the Vibrio Danubius (found in the Danube), the Vibrio Schuylkiliensis (found in Philadelphia by Abbott). They are all non-pathogenic.

[1] The student must not confound the Vibrio proteus with the Bacillus proteus, which is an entirely different organism.

SPIROCHETE.

The first spirochete described under the name of Spirocheta plicatilis by Ehrenberg and found in marshy water probably does not belong to the organisms classified today as spirilla, or spirochete. The most important pathogenic species are the following: *Spirocheta Obermeieri*, found in relapsing fever in man; *Spirocheta Duttoni*, found in African tick fever in man, and *Spirocheta pallida*, found in syphilis in man. There are also spirochete causing diseases of domestic birds and others, which have been found in mammals other than man, but which are probably not very pathogenic.

Spirochete in Man.—SPIROCHETA OBERMEIERI.—The first pathogenic spirillum was discovered by Obermeier in 1868 in the blood of persons suffering from relapsing fever. Though the role of bacteria in the production of infectious diseases was not yet well recognized at this early time, it was, nevertheless, believed that this organism was the cause of the disease. All spirochete known at present have resisted every attempt at artificial cultivation, and they are not as well known as most other pathogenic bacteria. It is now generally believed that the Spirillum Obermeieri is transmitted from sick to healthy persons through the bites of bedbugs. According to Novy and Knapp the spirochete of European and Indian relapsing fever are not identical, but different species.

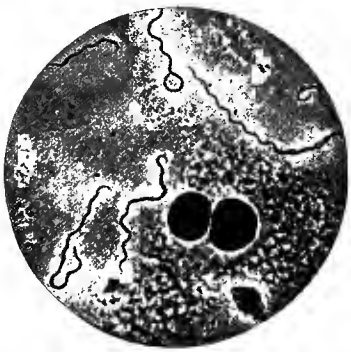
SPIROCHETA DUTTONI.—Tick fever, a disease of man prevalent in Equatorial Africa, was shown to be an infection due to spirochete. The first observations concerning its nature were made by Ross and Milne in Uganda and by Dutton and Todd in Eastern Congo. This disease is transmitted through the tick *Ornithodoros moubata*. Robert Koch, who studied the disease in German East Africa, demonstrated the presence of spirochete in the eggs as well as in the adult ticks. Novy has shown that the spirochete of African tick fever is a species distinct from the organism of European and Indian relapsing fever, and he has proposed the name of Spirocheta Duttoni in honor of Dutton, who lost his life while studying the disease in Africa.

SPIROCHETA PALLIDA.—The interest in the study of spirochete was enormously increased and undertaken by hundreds of investigators when Schaudin and Hoffmann reported that they had found an exceedingly fine, slender, long, and very typical spirocheta in the primary and secondary lesions of human syphilis. It was first named Spirocheta pallida, later Spironema pallida, and still later Treponema pallidum, but it is now most commonly known in literature as Spirocheta pallida, or the spirocheta of syphilis.

Spirocheta pallida is found in almost every primary syphilitic lesion; often in secondary but very rarely in tertiary lesions. It is a very slender spiral, from 4 to 14 micra long. It can best be seen in a living state, and unstained by the aid of the dark field illuminator.

It shows from six to fourteen windings or twists, and has generally a very regular corkscrew shape. It is very lively motile in a manner

FIG. 156



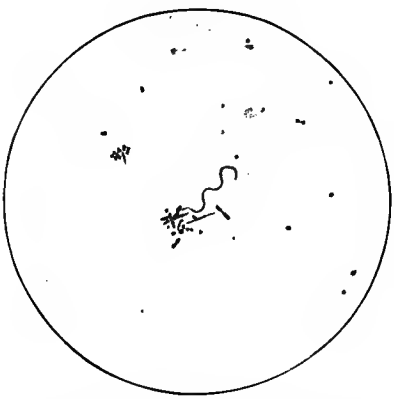
Spirocheta Obermeieri blood smear. Fuchsin. × 1000. (From Itzerott and Niemann.)

FIG. 157

Spirocheta pallida in the centre, several bacilli in the field. India-ink method. × 1000. (Author's preparation.)

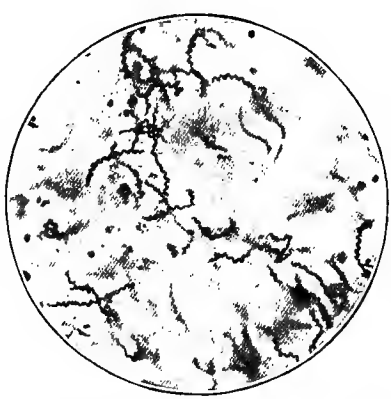
already described as common to all spirochete. It cannot be stained by the ordinary staining methods, but can be exhibited by the Giemsa stain. It is necessary to make a very thin smear and to fix the air-dry

FIG. 158



Spirocheta pallida, part of one which shows the typical spiral shape and unstained spaces in the protoplasm. Smear from primary syphilitic sore stained with Goldhorn's stain. × 1000. (Author's preparation.)

FIG. 159



Spirocheta pallida in liver of a case of congenital syphilis. Levaditi's silver impregnation method. × 1000. (Author's preparation.)

specimen for at least fifteen minutes in absolute alcohol. The ready-made concentrated Giemsa stain is diluted by adding one drop of the

stain to each cubic centimeter of distilled water. It is well to add a few drops of a one-tenth per cent. solution of carbonate of sodium and a few drops of glycerin to about 30 c.c. of the dilute solution. The stain must act for three or more hours, and the specimen is then washed in water and dried. If a precipitate has formed the slide or cover-glass should be washed rapidly in 90 per cent. alcohol and then again stained for some time with the dilute Giemsa stain without the addition of an alkali. The best method to show the Spirocheta pallida rapidly is the India-ink method, described in the chapter on Staining Technique. Spirilla found on the skin, mucous membranes, and in secretions of man which might be confounded with the Spirocheta pallida are the following: *Spirocheta refringens, Spirocheta balanitidis, Spirocheta buccalis, Spirocheta dentium,* and *Spirocheta pseudopallida* or *gracilis.* The first three are generally much coarser than the pallida, and stain blue with Giemsa stain, while the pallida stains pink. The last two, however, are almost as fine as the pallida, and as they also stain pink, they may be easily mistaken for it.

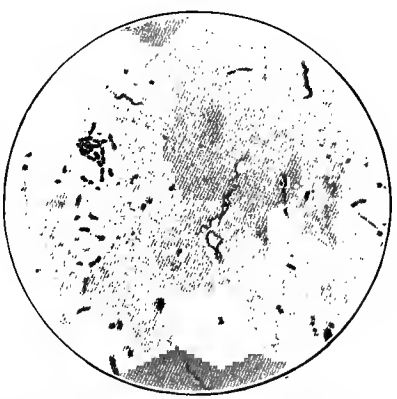
The question whether the Spirocheta pallida is really the cause of human syphilis is not fully settled: certain discrepancies still remain to be cleared up. The organism, however, is generally found in primary and secondary syphilitic lesions, and it is of great diagnostic value in recognizing the disease.

Spirochete in Birds.—Following the discovery of the Spirillum Obermeieri, certain spirochete pathogenic for birds were found, and later others in mammals and man. They were found in diseases of domestic birds, and resembled the Spirocheta Obermeieri, which causes recurrent fever in man. The first organism of this type was found in geese in 1893 by Sakharoff in Russia, and named accordingly Spirocheta anserina. The same bacterium was seen by Ducleaux in Tunis, likewise in geese. Marchoux and Salimbeni (1903) found spirochete in Brazil in chickens, and named them Spirocheta gallinarum. Since then spirochete have been reported from Rhodesia, India, Soudan, Algiers, Tunis, Cyprus, Martinique, Bulgaria, and the last report by Dodd comes from Queensland. Spirillosis of domestic birds, therefore, appears to occur over a widespread area. The disease causes fever, diarrhea, and emaciation, and either ends fatally within a few days or leads to recovery. Postmortem examination of dead animals shows enlargement of the spleen, enlargement and fatty degeneration of the liver, and sometimes fatty degeneration of the myocardium, with fibrinous deposits on the endocardium. The spirilla are found in the blood during the disease. They disappear, however, before death, and cannot be found in the cadaver; they also disappear in the case of recovery. A single attack protects against subsequent infection. The disease is conveyed from one animal to another through parasitic fowl ticks (Argas miniatus), in the body of which the organism can evidently remain alive for a long time. The disease can also be transferred by artificial inoculation of the

blood of infected birds into healthy ones. Young birds generally die after an artificial infection. An immune serum may also be prepared by repeated injections of blood containing spirilla into a horse.

Spirochete in Mammals.—Organisms of this type have been found by Theiler in the Transvaal in the blood of cattle simultaneously infected with Piroplasmata and Trypanosomata. The spirilla seen were slender and 20 to 30 micra long, and very similar to those described in birds. Laveran named this organism *Spirillum Theileri.* It is not known whether it is pathogenic or not. The observations of Theiler on cattle have been confirmed by Ziemann in the Cameroon and by Robert Koch in East Africa. These spirochete of cattle are transmitted through the bite of the tick *Rhipicephalus decoloratus,* which may at the same time transmit Texas fever, as shown by certain experiments of Laveran and Vallee. Theiler also found spirochete in sheep in the Transvaal, and he and other authors have a few times found the organisms in horses. Spirochete have also been seen in rats in India, and Nicolle and Comte found them in a common bat (Vespertilio Kuhli) in Tunis. The latter spirilla are 12 to 18 micra long, not more than one-quarter of a micron thick. They have pointed ends and divide by binary division at right angles to the long axis. The infection can be transmitted from sick to healthy animals.

FIG. 160



Spirocheta pallida, twisted and intertwined form, primary lesion of syphilis. Goldhorn's stain. × 1000. (Author's preparation.)

Are Spirochete Bacteria or Protozoa?—Extensive studies on the spirochete of relapsing and tick fevers and other organisms of this group have led Novy and Knapp to agree with the conclusions previously drawn by Carter, Norris, Martin, Borrel, R. Koch, and others that spirochete are bacteria, *i. e.,* vegetable microörganisms, and not Protozoa, as Schaudin and other investigators have believed. In the examination of living spirochete, Novy and Knapp failed to observe the presence of an undulating membrane with a flagellum

as encountered in trypanosomes.[1] They found the contents of the spirochetal body perfectly homogeneous, without any indication of a nucleus and a blepharoblast. The flagella had the characteristics of bacterial organs of locomotion and not those found in protozoa. On the Spirocheta Obermeieri they were able to demonstrate a slender whip as long as the body of the organism, wavy, like the flagella of other bacteria, and not coarse and thick like the flagella of trypanosomes. They observed evidences of transverse binary division at right angles to the long axis both in living and stained preparations. They also ascertained that spirochete included in very thin collodion sacs and exposed to the action of running distilled water or directly mixed with distilled water showed plasmolytic changes like bacteria and not like the much more susceptible and delicate protozoa, particularly trypanosomes. They further demonstrated that spirochete are not as susceptible to higher temperatures as trypanosomes, but act more like bacteria, and do not, under elevated temperatures, change their shape and form like trypanosomes. In their rapid method of multiplication when injected into susceptible animals and in the production of immunity spirochete likewise act like bacteria and not like trypanosomes. The author has had opportunity to study Spirocheta pallida extensively both in the live state with the dark field illuminator and in stained and silvered preparations, and he likewise believes that spirochete are bacterial and not protozoan organisms, that they do not possess an undulating membrane, a nucleus, or a blepharoblast like the protozoan trypanosomes, and that they divide at right angles to the long axis like other bacteria. Anyone who has studied a large number of stained specimens of Spirocheta pallida must have repeatedly seen forms where the division in the middle of the long axis was clearly indicated and almost complete. The forms mistaken for spirocheta dividing by splitting parallel with the long axis are simply a pair of intertwined spirals and not a dividing organism.

QUESTIONS.

1. Name and give the characteristics of the two groups of spiral bacteria.
2. Describe the type of motility of vibrio and of spirocheta.
3. Where was the vibrio of Metchnikoff first found? What disease does it produce?
4. Describe the disease caused in chickens by the Vibrio Metchnikovi. Describe the pathologic lesions.
5. What names have been given to this disease in chickens?
6. Describe the morphology of the Vibrio Metchnikovi.
7. Describe its cultural properties.
8. Does it always liquefy gelatin?
9. What is the difference in animal inoculations of the Vibrio Metchnikovi and of the vibrio of Asiatic cholera?
10. What animal diseases are caused by the vibrio of Asiatic cholera?

[1] The student should compare the description as given in the chapter on Trypanosomes, with the facts here given as to the morphology of spirochete.

11. What are the characteristic cultural differences between the vibriones of Asiatic cholera and of Metchnikoff?
12. What is the test for the presence of nitrites and indol in culture media?
13. Name and describe some other non-pathogenic vibriones.
14. What was the first pathogenic spirocheta discovered? What disease does it produce? How is this disease spread?
15. What is the cause of African tick fever. How is it spread?
16. Describe the morphology, staining, and cultural properties of Spirocheta pallida.
17. In what disease is it found? How can its presence best be demonstrated?
18. Describe some diseases caused by spirocheta in domestic birds. How are those diseases spread?
19. State in what mammals spirochete have been found and what is known about their pathogenesis.
20. Discuss the question whether spirochete are protozoa and near relatives of trypanosomes or not.

CHAPTER XXXIV.

THE BACILLUS LACTIMORBI OF TREMBLES.

Occurrence.—The disease known as milk sickness, sick stomach, swamp sickness, tires, trembles, slows, etc., is an affection of cattle, and occasionally of sheep. It has been observed in the United States for over one hundred years, and apparently has never been described in any other part of the world. It was formerly more frequently mentioned, and has evidently decreased considerably. An affected animal is lifeless, tired out on the slightest exertion, and the muscular weakness is manifested by trembling, which characteristic symptom has led to the designation "trembles." In a more advanced stage there is stiffness of the joints, great weakness and the animal is unable to get up after it has once fallen to the ground.

Pathologic Lesions.—Jordan and Harris describe the pathologic lesions of the disease in cattle as follows: There are no characteristic external changes, but upon opening the body the smell of acetone can often be detected. Occasionally a small quantity of clear yellow fluid is present in the pleural cavity. The lungs are edematous. The visceral layer of the pericardium, chiefly along the course of the cardiac veins from base to apex, and around the roots of the large vessels, shows ecchymotic spots, which are also occasionally seen in the parietal layer of the peritoneum. The liver is much enlarged, of a purple red color, and much congested; it appears mottled in consequence of the presence of areas of fatty degeneration, of which it shows marked evidences on section; the consistency of the organ is much diminished. The spleen is not enlarged. The kidneys are enlarged, congested, and sometimes show evidences of parenchymatous degeneration. The mucosa of the small intestines is congested, and shows ecchymotic spots, and the jejunum and the upper part of the ileum are covered by a yellowish, very tenacious mucus.

The disease appears to be usually contracted by grazing cattle or sheep which have entered an infected territory. It may then be communicated to man through raw milk or butter, or through raw or insufficiently cooked meat; dogs and cats may also become infected. The mortality in man is claimed to be high, but this is probably incorrect, because, as a rule, severe cases only are recognized while the milder cases have escaped notice.

Morphology and Staining Properties.—Jordan and Harris isolated an aërobic organism which they called *Bacillus lactimorbi* from the internal organs of cattle dead from trembles. They describe it as follows: In cover-glass preparations made from the juice of the

organs the bacilli are longer and more slender than the colon bacillus; they stain occasionally unevenly with methylene blue. In preparations made from cultures grown on *agar* at 37° C., the organism is found to be a rod, a little smaller than the anthrax bacillus, occurring singly and in pairs and in occasional filaments. As a rule, the rods, at the end of twenty-four hours' incubation, do not stain deeply with methylene blue, even if the solution be slightly heated, but at one or both poles and at the centre of each rod metachromatic granules are found which take on a reddish or purple tint. In young cultures the bacilli are Gram positive. *Spore formation* occurs after twenty-four hours' incubation, the spores may be first oval, but when completely mature they are round. They lie near one end of the rod. The organism is motile and possesses ten to fifteen peritrichous flagella, which can be demonstrated by van Ermengem's method.

Cultural Properties.—The cultural characteristics of the organism are as follows: On *agar slants* incubated at 37° C., at the end of twenty-four hours, the surface is more or less irregularly covered by a delicate veil-like growth, which is more profuse at the end of from forty-eight to seventy-two hours; on some cultures it may eventually take on a semiviscid character. The color is grayish, moist, smooth, and glossy; there is no pigmentation of the growth itself or of the medium. There is no gas, the condensation water-growth is heavy, gray white in color; no odor being present. No gas is formed in *glucose agar cultures*. In *bouillon*, at the end of twenty-four hours, no growth except sometimes a slight clouding at the surface, may be noticeable. At the end of twenty-four hours a well-formed pellicle, which will sink if the tube is agitated, appears on the surface. The remainder of the medium is feebly clouded, and there may be a fine semiflocculent sedimented growth at the bottom of the tube. *Milk* becomes alkaline after a number of days, but coagulation does not occur; there is no growth on *potatoes*. In *gelatin stab cultures*, kept at 20° C., the growth begins to appear at the end of forty-eight hours, but is not well developed until at the end of seventy-two hours. There is little or no surface growth, but down the stab to the extent of about 2 centimeters, a delicate, smooth, gray growth appears. At the end of a week the surface growth has extended as a fairly well-developed film of a whitish color, smooth, moist, and glossy; at times the first evidence of liquefaction may now be detected, but this is not well established until the tenth day, when the medium is slightly fluid just beneath the surface growth. As time passes liquefaction slowly progresses. The growth in gelatin leads to a more or less well-defined putrescent odor. *Agar plate* colonies at the end of twenty-four hours' incubation at 30° to 37° C. resemble those of the Streptococcus pyogenes, but with this difference, that they show a tendency to spread out in a film, particularly if the agar is freshly made; in the latter case the whole surface of the plate may be covered. In *gelatin plates* grown at 20° C. the colonies make their appearance at the end of from forty-three to seventy-two hours, and resemble at

first those of streptococci, but at the end of the fourth day they appear more vigorous, and the surface colonies partake more of the characteristics of Staphylococcus albus, although the color is of a yellowish-white character.

The *thermal death point* of the organism was ascertained to be five minutes' exposure to 55° C. for the vegetative form, fifteen minutes at 100° C. for the spores.

Agglutination tests of the serum of cattle and man with the Bacillus lactimorbi did not furnish any uniform results. Some sera would agglutinate some stems of bacilli in dilution of 1 to 50 or 200; others would not be agglutinated, and some stems of the organism could not be agglutinated by any sera.

Jordan and Harris found the Bacillus lactimorbi, or an organism not distinguishable from it by any of the tests applied, in the soil of regions where milk sickness has never been known; they found it in normal cow dung, on various grain and forage plants.

Luckhardt isolated an identical organism from dried alfalfa from Wisconsin, from the same material from Illinois and Indiana, and from four weeds collected by Jordan and Harris in the Pecos Valley in New Mexico, where they first studied the disease. Of six dogs inoculated with cultures of these stems of bacilli obtained by Luckhardt, two showed in a slight degree the symptoms observed in milk sickness. Luckhardt concludes from his work with the Bacillus lactimorbi that it is remarkable that if this bacillus be the cause of milk sickness it should have so wide a distribution in milk-sick and non-milk-sick regions. It is also apparent that loss of virulence occurs after a time in the races of the organisms isolated, and that no feeding experiments have so far yielded uniformly any well-defined pathologic picture of the disease.

The animal experiments of Jordan and Harris have likewise lacked uniformity and definiteness in their outcome. On the whole, it cannot yet be considered as established that the Bacillus lactimorbi is indeed the cause of trembles. Some of the results of the animal experiments made with the blood of dead animals and with pure cultures of the bacillus rather point to the possibility of an ultramicroscopic virus.

QUESTIONS.

1. What other names have been given to milk sickness in cattle?
2. Where has the disease been encountered?
3. What are its most characteristic symptoms?
4. Describe the pathologic changes found after death from this disease.
5. Is the disease communicable to man, and how?
6. Describe the morphology and staining properties of the Bacillus lactimorbi.
7. Describe a culture on agar.
8. How does the organism affect milk when grown in it?
9. Describe its growth in gelatin.
10. What is the thermal death point of the vegetative form and of the spores of the organism?
11. What has been the result of agglutination tests with the organism?
12. Where has the Bacillus lactimorbi been found outside of the bodies of animals dead from trembles?

CHAPTER XXXV.

A LOCAL EQUINE DISEASE AND A BACILLUS OF THE SUBTILIS GROUP.

In the neighborhood of Lake Winnebago and in other places in Wisconsin a disease among horses characterized by a rather rapid onset has been frequently observed in the late summer or early fall. The animal appears to be perfectly well in the evening, the following morning it refuses to eat or drink, inclines the head downward, breathes very rapidly, has a rapid pulse of about 80, and a temperature ranging from 104° to 107° F. The stools are first loose, and a profuse diarrhea, which reaches its maximum in about three days, develops rapidly. Sometimes the bowels are not loose but rather constipated, and then pulmonary symptoms develop. The animals so affected generally die.

The author, in conjunction with Dr. O. N. Johnson, of Appleton, Wis., in August, 1908, examined bacteriologically a number of cases of this affection occurring in Dr. Johnson's practice. Blood was obtained from the jugular vein of sick animals under aseptic precautions in 8 cases and immediately distributed to culture tubes and flasks. In 5 cases an identical organism was obtained. It was a rather large, lively motile, Gram-positive bacillus, which grew best at room temperatures, less rapidly at the incubator temperature (37° C.). On *agar* it formed a white, moist, shining, rather rapidly spreading growth; *gelatin* in stick cultures was rapidly liquefied, with the formation of a funnel-shaped zone of liquefaction; it produced acid in litmus milk and coagulated it after seventy-two hours; on *glucose agar* it did not grow as well as in plain agar along the stab, but grew well on the surface and did not produce any gas. In *bouillon* turbidity it was produced rapidly, but no pellicle was formed on the surface. *Spore formation* occurred in various media after twenty-four hours' incubation at 37° C. One of the stems examined had not coagulated milk after seventy-two hours and another stem formed a dry, hard pellicle on bouillon. In other respects the action of the five stems was identical. On the whole, the organism appeared to be a member of the subtilis group, but it seems hardly possible that it was an outside contamination, as it developed in the blood inoculations from five out of eight horses examined.

Emulsions of the organism raised on agar slants and prepared with physiologic salt solution were injected intraperitoneally into a number of guinea-pigs. During the first three or four days no visible

effect was produced in the inoculated animals. On the morning of the fifth day guinea-pigs inoculated from cultures Nos. 1, 2, and 3 (each from a different horse) were found dead. On the seventh day the guinea-pig inoculated from stem No. 5 was found dead. Autopsies on the dead animal showed no typical lesions. Cultures were made from the heart's blood of the four dead guinea-pigs; in 3 cases an organism identical with the one injected was obtained but the cultures soon died out. One which remained alive after several transplants was inoculated subcutaneously into a healthy horse. It failed to produce any general reaction, the temperature of the horse was taken regularly, and remained normal for a number of days. A slight local reaction developed at the site of the injection (neck), but this disappeared after a few days. In the bacteriologic part of this investigation the author was assisted by Dr. Conrad Jacobson. No definite conclusion as to the relation of the bacillus obtained to the disease can be drawn from the above results. Further and more extensive studies are necessary.

CHAPTER XXXVI.

LOWER HYPHOMYCETES—TRICHOMYCETES—LEPTOTHRIX—CLADOTHRIX—STREPTOTHRIX AND ACTINOMYCES.

HYPHOMYCETES.

THE organisms discussed in the preceding pages have been considered chiefly from the point of view of being the cause or etiologic factor of disease in domestic animals and man. All of them are exceedingly simple, unicellular, vegetable microbes, and they are known as bacteria or schizomycetes. The term schizomycetes denotes *fission fungi* and is descriptive of the characteristic mode of binary division or fission common to all organisms belonging to the bacteria. True branching[1] does not occur among the latter, while the higher fungi, eumycetes or hyphomycetes, do form genuine branches. For this reason they may be defined as vegetable microörganisms composed of elongated or filiform cells, which form genuine branches and which multiply by special organs or cells called spores. Their development is characterized by the formation of true branches from the original cell or main trunk, and the latter and the branches form one continuous mass of protoplasm. The branching may be a comparatively simple arrangement as in the lower eumycetes, or it may be relatively complicated with a vast network of dichotomous and pseudodichotomous divisions as in the higher eumycetes. The term true dichotomous division designates a division into two equal branches, while pseudodichotomous division indicates that one branch, which is continued as the principal stem, sends out a lateral, often smaller branch.

When the hyphomycetes or, as they are commonly called, the moulds are studied a differentiation of the organism into two parts which perform different physiologic functions can be distinguished. One part, the *mycelium*, presents itself as a more or less branched mass which serves for the nutrition and preservation of the individual organism, while the second part, known as the *fructification organ*, produces the spores and serves for the preservation of the species. The entire soft cellular structure, without any wooden fibers, of which the whole body of the mould consists, is known as the *thallus*.

[1] Bacteria like the tubercle bacillus are not taken into consideration at this place, notwithstanding the fact that this organism sometimes forms true branches, indicating, perhaps, that it should be classified among the lower eumycetes rather than among the bacteria. Since such organisms have always been classified among the bacteria in medical considerations, it is not desirable to make any change.

According to Lafar, the mycelium may, therefore, be defined as that portion of the thallus of the mould which is spread out on the culture soil and which derives from it the nutrition necessary for the organism. The mycelium arises from a spore which has been implanted upon or within a favorable culture soil. From the spore one or several *germ sacs* are formed; these grow elongate, divide, and form the individual *hyphen* or *mould filaments* which in their entity compose the mould mycelium. The latter always grows at the external ends of the individual hyphens and not at the end which originally arose from the spore. In this respect the eumycetes differ from the schizomycetes, which grow at both ends and in multiplication divide in the middle. In the study of the mycelium two types are noted, one in which the whole mass, much branched and complicated as it may be, consists of a *single cell* only; the other in which the hyphens, or filaments, are divided by septa into cylindrical segments, which by their union form the hyphens. Moulds of the first type are classified as *phycomycetes;* those of the latter as *mycomycetes.* The fructifying organ is also developed in all hyphomycetes, though in the lowest forms it may be rather rudimentary and inconspicuous. The function of the fructifying organ is the production of cells from which new individuals can be formed. Such cells of lower plants, as has already been stated in the consideration of bacteria, are called *spores.* A bacterium, however, almost invariably forms a single spore only, while hyphomycetes generally form a large number of spores.

Spore Formation.—Four different varieties of spore formation are recognized in hyphomycetes, namely:

1. Endospores, or gonidia.
2. Zygospores.
3 Exospores, or conidia.
4. Chlamydospores, or gemmæ.

The first three types of spores are formed by the fructifying organ, except among the very lowest type of hyphomycetes, where the cells of the mycelium form the spores in their interior without any preliminary differentiation. This mode of formation is like that in bacteria, only that it is generally a multiple and not a single spore formation.

1. Endospores, or Gonidia.—In the formation of the fructifying organ one or several hyphens arise from the mycelium and grow upward, becoming straight and much stronger than the ordinary mycelial hyphens or filaments. This stem is called the *fruit bearer,* or *fruit carrier.* Its upper free end becomes globularly or elliptically enlarged, and in this extremity the spores are formed after it has become separated from the remainder of the stem by a straight or curved septum. This free round or oval separate end forming the spores is now called a *sporangium,* while the stem which carries the latter is often called the *columella* (the little pillar). This is the mode in which endospores or gonidia are generally formed.

2. Zygospores.—This variety of spores is formed as follows: The outer, free ends of two hyphens, or filaments, swell up and become club-shaped. The clubs touch each other and the membranes separating them become fused. The united clubs at the same time become divided from the rest of their hyphens by two septa. In this way two separate cells touching each other are formed. These are called *copulation cells*, or *gametes*, while the upper part of the hyphens which carry the latter are known as the *carrying cells*, or *suspensors*. The partition wall, or septum, between the two gametes touching each other finally become dissolved, and from the contents of the two copulation cells the spore, known as a *zygospore*, is formed. Spores are also sometimes formed from a single hyphen without the union of two gametes, and this variety of spores is called *azygospores*, or *parthenospores*.

3. Exospores, or Conidia.—These may be formed by either of the following ways: In some hyphomycetes the fruit bearer at its upper extremity forms a round end which becomes separated from the rest of the filament by a septum. The cut-off segment then assumes a round or oval shape, forming thus the first spore. The end of the hyphen to which the first spore adheres repeats the process, and a second spore is formed situated between the first spore and the end of the hyphen. This process continues, and gradually a *row of spores*, of which the outermost is the oldest and the innermost the youngest, becomes attached to the fruit-bearing hyphen. In the other method of spore formation the first spore is formed in the manner just described, but then it itself divides, and this process continuing, a row of spores is formed in which the outermost is the youngest and the innermost the oldest. The first mode of spore formation proceeding from the inner end of the row is called *basipetal conidia formation;* the second, in which the new spores are formed at the outer end, is known as *basifugal conidia formation.*

Chlamydospores, Oidia, or Gemmæ Formation.—In this form of spore formation a fruit bearer proper is not formed, but the filaments of the mycelium break up into small segments. Each of these when falling upon a fertile soil may form a new hyphomycete individual. This mode of spore formation was first observed in a fungus known as *Oidium lactis*, and for this reason it is also called *oidia formation.* The small segments arising in gemmæ formation often assume a more marked spore character by surrounding themselves with a tough, tenacious, protecting membrane.

Resistance of Spores.—The resistance of spores of hyphomycetes is generally like that of the spores of bacteria, and like them they are more resistant than the adult form of the organism. As a rule, however, their resistance is not as great as that of bacteria, though some may withstand drying out for many years. Hansen has shown that the spores of certain common moulds survived after a desiccation of from eight to twenty-one years. Pasteur has shown that the conidia of

the common blue mould (Penicillium glaucum) while killed by boiling water can withstand dry heat at 120° C. for some time.

Classification.—In considering now some of the pathogenic microorganisms of a higher development and more complicated morphology than the bacteria, the classification proposed by Lackner-Sandoval and adopted by Petruschky in his contribution on pathogenic trichomycetes in Kolle and Wassermann's *Manual* will be followed. Petruschky divides the hyphomycetes into the trichomycetes (hair fungi) and the higher hyphomycetes. The former are again subdivided into (1) leptothrix, (2) cladothrix, (3) streptothrix, and (4) actinomyces.

The first two genera are nearly related to the bacteria or schizomycetes, while the latter two are clearly members of the order of hyphomycetes or eumycetes.

1. LEPTOTHRIX.—These present themselves as stiff, slender filaments which do not show any branching and on which dividing processes can rarely be observed.

2. CLADOTHRIX.—These are filaments with pseudobranching. The branching effect is produced by a protrusion of the protoplasm through the membrane and a rapid breaking up of the filaments into short rods which assume the character of bacilli.

3. STREPTOTHRIX.—This genus forms a true branching mycelium with septa formation and the production of fruit-bearing filaments. The latter break up into short segments which form chains of exospores, or conidia.

4. ACTINOMYCES.—This is really a streptothrix and it systematically does not differ from the latter. It shows, however, the peculiar property when invading tissues as a pathogenic microörganism of forming in the pathologic lesions very characteristic stars, composed of the ends of swollen hyphens or filaments, arranged in rosette form. On this account the organism has received the name of ray fungus, or actinomyces, and it is well to retain it and classify separately the pathogenic streptothrices showing this property.

1. *Leptothrices.*—These are represented by a species frequently found in the mouths of persons and known as Leptothrix buccalis. The organism is, as a rule, a harmless saprophyte, but it has sometimes been found as the cause of acute or chronic inflammations of the pharynx in man. Piana has observed a case of pleuritis in a dog caused by an organism similar to or identical with the Leptothrix buccalis.

2. *Cladothrices.*—Cladothrix asteroides is the name given by Eppinger to an organism found as the cause of pseudotubercular lesions in guinea-pigs. It is described as consisting of filaments with pseudobranches. The filaments later break up into segments of cuboidal and bacillary shape. The organism grows rapidly at blood temperature, poorly under anaërobic conditions, and does not liquefy gelatin. It forms round colonies, which subsequently become con-

fluent and form a corrugated, tenacious, orange-colored membrane. The organism does not coagulate milk.

Cladothrix canis was found by Rabe in cases of peritonitis in dogs and in purulent processes of the skin and subcutaneous connective tissue. In pus and softened lymph glands, the organism appeared as a conglomeration of filaments. The latter were straight, angular or wavy, branching, and also found broken up into bacillary segments. Rabe considered the organism the cause of the pathologic lesions in which it was found. It is uncertain whether the organism is really a cladothrix or whether it is not identical with the Streptothrix canis described below.

3. *Streptothrices.*—These have been found a number of times in pathologic conditions of domestic animals and man. In the latter streptothrix infection has been described by Eppinger, Petruschky, Aoyama and Miyamoto, MacCallum, Flexner, Norris and Larkin, Wharthin and Olney, Butterfield, and others. The most interesting streptothrix infection in man is that known as mycetoma, or madura foot, of which two varieties, a brown and a white occur. The latter is due to the Streptothrix maduræ.

4. *Actinomyces.*—The streptothrix which forms the typical rosettes in the pathologic lesions which it causes is described fully in Chapter XXXVII.

PATHOGENIC STREPTOTHRICES.

Streptothrix Farcinica.—The disease known as farcy in cattle, lymphangioitis farciminosa bovis, farcin du bœf (French), "Hautwurm des Rindes" (German), was first described by Sorillon (1829) as being frequently seen in France. Later French writers have also repeatedly mentioned it, but the disease is now rare in France. Nocard, in 1887, discovered a streptothrix as the cause of the disease.

The pathologic changes are generally found in the extremities of the animal, on the interior surfaces of which, along the superficial veins, non-painful cords and nodules of very firm consistency appear. Later they sometimes become softer, even fluctuating, and discharge upon section, a whitish, soft, non-fetid mass. Sometimes fluctuating nodules open spontaneously, but the wounds, as a rule, soon heal again. Generally the lesions do not become softened, but remain permanently composed of a firm, tough, fibrous, connective tissue. The disease may exist a year or longer without disturbance of the general condition. Later it occasionally leads to emaciation and even cachexia and death.

Farcy in cattle is caused by the *Streptothrix farcinica,* which presents itself in the lesions as branching filaments. The organism is aërobic, and grows best between 30° and 40° C. In *bouillon* it forms whitish granules which soon fall to the bottom of the tube. On the surface round, dirty gray masses are found, which in reflected light

have a greenish and dusted appearance; they are not moistened by water which runs off as from a fatty substance. The organism grows best in alkaline or neutral bouillon; a very slight acidity, however, does not inhibit development; the reaction of the medium is not changed. On *agar* there develop small, round, opaque colonies with thickened margins and an uneven surface, which later looks as if dusted over. Finally the surface becomes covered with a thick, uneven layer.

The appearance on *coagulated blood* serum resembles that on agar. The growth, however, is not so rapid. On *potatoes* dry, uneven, pale yellow colonies, with elevated margins, are formed. The organism grows in *milk*, but neither produces acid nor coagulates it. The organism is fairly resistant; it is killed after an exposure of ten minutes to 70° C. When inoculated intraperitoneally into guinea-pigs it produces pseudotuberculous lesions; subcutaneous inoculations into cattle and sheep produce lesions like those of farcy found under natural conditions. According to Nocard's experiments, rabbits, dogs, cats, and equines are not susceptible to the Streptothrix farcinica.

Streptothrix Canis.—A number of cases of pleuritis and peritonitis with slight elevation of temperature and the formation of a reddish-brown, cloudy exudate in which white granules can be seen with the naked eye have been observed in dogs. The pleura and peritoneum are sometimes studded with fibrous appendages or villi, while the lungs may contain hard, gray nodules of the size of a pea. In the exudate and the tissue lesions *conglomerations of slender filaments* are found. The organism has been studied by Bahr. The streptothrix filaments can be stained by Gram's method and sometimes show club-like swellings at their free ends. The Streptothrix canis, as it has been called, grows on artificial culture media at incubator temperature, first only aërobically, later also anaërobically. In the depth of the *agar* mulberry-like colonies composed of long, dividing filaments, sometimes thicker at the ends are formed after three or four days; threads broken up into segments are likewise seen. Pure cultures inoculated intraperitoneally into mice produce purulent abscesses. Rabbits infected subcutaneously develop nodules up to the size of a hazelnut. Dogs can also be infected subcutaneously, and the streptothrix is found in the nodules and abscesses which develop.

Actinomyces Bicolor.—Another streptothrix has been found in a case of multiple brain abscesses in a dog. From the pus Trolldenier obtained in pure cultures a streptothrix or actinomyces which formed on *agar* colonies which were yellow in the centre and white at the periphery. The organism which proved pathogenic for mice, guinea-pigs, rabbits, and dogs was called Actinomyces bicolor.

Streptothrix Capræ.—In a goat suffering from a pseudotubercular affection of the lungs Zschokke found a streptothrix which was not only Gram positive but also acid fast. The organism was more thoroughly studied by Silberschmidt, who ascribed the following

properties to it: Very thin, wavy filament, pseudodichotomous division, some filaments broken up into segments; on the surface filaments broken up into coccus-like bodies (spores). The organism grows rapidly under aërobic (not under anaërobic) conditions, both at room and at incubator temperature. The growth is light brown reddish and appears as if covered with a white dust. The colonies on *agar* are elevated with a depressed centre, uneven, corrugated, and powdered with a white dust. Sometimes they are white and velvety and look like the growth of a higher mould. In *bouillon* disk-like, dry, isolated colonies are formed on the surface; a complete pellicle does not form. The sediment is granular. Growth on *potatoes* is rapid; first reddish, then white. On *milk* a reddish pellicle is formed; coagulation does not occur. Subcutaneous inoculation of pure cultures produce abscesses in guinea-pigs, rabbits, and mice. Intraperitoneal or intravenous injections produce pseudotubercular lesions.

A streptothrix of the same type, also acid fast and leading to pseudotubercular lesions in the lungs of a young man suffering and dying from diabetes, has been described by Butterfield. Since the organism was not obtained in pure culture and no animal experiments were made, it is impossible to say whether it is identical with Streptothrix capræ or not.

QUESTIONS.

1. What are the characteristics of hyphomycetes or eumycetes?
2. In what features do they differ from schizomycetes?
3. What is the difference between true and false branching?
4. What is the difference between dichotomous and pseudodichotomous branching or division?
5. What is a thallus?
6. What is a mycelium?
7. How does the mycelium grow and extend?
8. Differentiate between phycomycetes and mycomycetes.
9. What is the function of the fructifying organ of hyphomycetes?
10. Name the four types of spores formed by hyphomycetes.
11. Describe the characteristics of the four different types.
12. What are copulating cells? What other name are they known by?
13. What are suspensors?
14. What is meant by basipetal conidia? What by basifugal conidia?
15. Why is chlamydospore-formation also called oidium-formation?
16. Discuss the resistance of spores of hyphomycetes.
17. What are trichomycetes? Why so named?
18. Name the four genera of the family trichomycetes.
19. Describe the characteristics of the four genera.
20. Name a species of leptothrix. Where found?
21. Name some species of cladothrix.
22. Have streptothricæ been found as the cause of human diseases?
23. Why are actinomyces considered separately from streptothricæ to which they belong?
24. Describe the Streptothrix farcinica and the lesions which it produces in cattle.
25. Describe the morphology, cultural properties, and pathologic lesions of Streptothrix canis.
26. Describe the morphology, cultural properties, and pathologic lesions of Streptothrix capræ.

CHAPTER XXXVII.

ACTINOMYCOSIS.

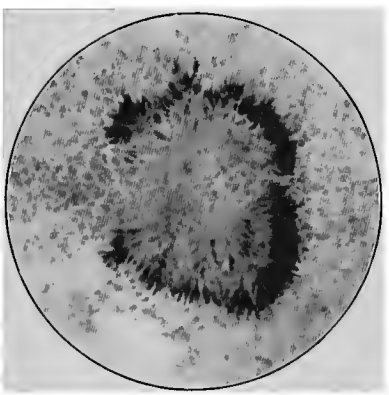
Occurrence and Historical.—Actinomycosis is a chronic infectious disease occurring principally in cattle and swine, also in man and more rarely in horses and sheep; occasional cases have been observed in deer, elephants, dogs, and cats. It is caused by a microörganism known as the actinomyces (actis ray; mykos, fungus), or ray fungus. Its growth in the tissues of susceptible animals leads to the formation of frequently large, granulomatous, and fibrous tumor masses. The disease in cattle has been known for a long time as lumpy jaw, wens, cancer of the tongue, osteosarcoma, etc. It is now known that Langenbeck, as early as 1845, saw the characteristic ray fungi in a human affection (then mistaken for osteosarcoma). In fact, he gave a picture of the organism, however, without recognizing its nature. In 1848 Lebert again furnished drawings of the ray fungus, and in 1868 Rivolta described it clearly from a case of lumpy jaw in cattle. In 1876 Bollinger recognized the parasitic nature of the actinomyces, and Hartz, a botanist, studied it from the biologic standpoint. A little later the etiology of the disease in man was established by Israel. Actinomycosis is distributed over the entire world, and the fungus is undoubtedly very prevalent in nature, where it is found on grasses. It is probably always contracted by the entrance of the bearded grains of barley, oats, wheat, etc., which carry the fungus into a wound of the buccal or respiratory mucosa. In fact, such evidently infected vegetable parts are very frequently found in actinomycotic lesions, particularly in those of the tongue in cattle. As early as 1882 Johne observed barley hulls infected with actinomyces in the tonsils of hogs, and he expressed the belief that grains of cereals acted as the infection carriers of the disease. It is not yet definitely settled whether actinomycosis in man and animals is due to the same species of ray fungus or whether there are several species or at least several varieties. There is no evidence that actinomycosis is ever spread by direct or indirect contagion from animal to animal or from animal to man; it is, so far as is known, only contracted through infected vegetable parts gaining entrance into the tissues of a susceptible being.

Pathologic Changes.—The ray fungus after entering into and multiplying in the body leads to a chronic inflammatory reaction with considerable new formation of tissue. The granulomatous masses may assume a very large size and appear like true neoplasms or

tumors. It is claimed that the ray fungus itself never leads to suppuration and that pus appears only after the inflammatory tissue has secondarily become invaded by pyogenic microörganisms. In cattle the disease generally begins at the head, and the lower jaw is the part which is apparently first affected, but actually it is the posterior part of the tongue, the region of the papillæ circumvallatæ, which is primarily affected. This region has generally been found infected in every case of actinomycosis of the head, and even when lumpy jaw had not yet developed, careful microscopic examination has frequently shown invasion of the tongue.

The most common tongue affection in cattle is in the form of *ulcerations* on the back of the tongue; the loss of substance is either round, oval, or band-like in shape. The margins of the ulcers are elevated, irregular, and ragged. These ulcers are frequently covered by hairs and vegetable fragments. If a section is made through the ulcerated surface, grayish-white nodules are seen extending into the muscular portion of the organ. These actinomycotic foci contain a greenish-yellow, tenacious, sticky material in which may be seen grayish, yellowish, or brownish granules, presenting masses of the fungi. Sometimes the tongue contains fistulous tracts. In other cases the surface and the muscular substance of the tongue shows a smaller or larger number of nodules, of peanut to hazelnut size. These have sometimes broken through the surface and then exhibit sharply defined openings. When such actinomycotic nodules have existed for a long time the connective tissue of the tongue is much increased and the organ much enlarged and very tough and hard in consistency, often as hard as a board. This is the so-called *wooden tongue* of actinomycosis.

FIG. 161



Actinomycosis. Section of granulation tissue of jaw of cattle. Carmin stain, Gram gentian violet. × 400. (Author's preparation.)

In the *maxillary bones* the disease arises from the alveoli of the teeth or from the periosteum. When the alveoli are the starting point of the infection the normal bone gradually becomes replaced by a soft, sarcoma-like mass, and while bone substance becomes necrotic and is more or less removed new bone is simultaneously formed by the periosteum in consequence of an *osteoplastic* (bone-forming) inflammatory process. The new-formed bone may again be broken through by the granulomatous masses and these may protrude externally through the skin, or internally into the cavity of

the mouth. In the interior of the granulation tissue which has been invaded and partly replaced the osseous bone substance is still present in the shape of a honeycombed network with wide open spaces. As the granulation tissue invades the maxillary bone more and more it often loosens teeth and lifts them out of their alveolar position. The gums also become involved and exhibit larger or smaller granulomatous masses, which may ulcerate extensively. The condition of the maxillary bones of cattle which have been extensively invaded by actinomycosis generally resembles that of bones which have become the seat of an osteosarcoma, and hence the disease was formerly mistaken for such a bone tumor. Actinomycosis starting from the periosteum of the maxillary bones generally leads to much denser, more solid, more fibrous tumor masses. In the mouth cavity actinomycotic masses frequently have the shape of *mushrooms* and *cauliflower excrescences.*

FIG. 162

Actinomycosis of the inferior maxillary bone of cattle.

Primary actinomycosis of the esophagus, the lower gastro-intestinal tract and the respiratory tract in cattle, is rare; the liver, however, is frequently full of actinomycotic abscesses in advanced cases.

The actinomycotic tumor of the head of cattle after having grown to a certain size frequently leads to superficial ulcerations. It then presents an uneven ulcerated surface from which granulomatous cauliflower masses may protrude. Such ulcerations discharge a yellowish, creamy, or very tenacious, gluey pus. In this pus the typical grayish-yellow or brownish granules of the ray fungus are generally found.

Subcutaneous actinomycosis in cattle is not always confined to the head, but is also found in the back, legs, and thighs. In swine actinomycosis frequently finds entrance through the tonsils or through the nipples, the latter being injured when the animals are kept in

stubble fields. In horses the disease has been observed in the tongue, lymph glands, etc. A fibrous cord in a horse, seen in 1908 by the author in a dissecting-room subject, was found to be due to actinomycosis. Cattle and hogs likewise contract actinomycosis from castration wounds.

In man, as in cattle, the ray fungus disease generally makes its entrance through wounds of the mouth. It has been known to follow the habit of chewing grain or picking the teeth with straws. Pieces of barley and pieces of straw have been found in decayed teeth from which the disease evidently started. In man, actinomycosis frequently begins in the intestines, where also its principal lesions develop and from which place it generally invades the liver.

Microscopically, parts infected with actinomyces show a granulation tissue composed of lymphoid and epithelioid cells, and also polynuclear giant cells. The latter are generally less regular in outline than those found in tuberculosis. External to the cellular tissue a zone of fibrous tissue is found. Actinomycotic tissue also shows a more or less pronounced infiltration, with ordinary polynuclear leukocytes; basophilic plasma cells are likewise seen.

Systematic Classification and Morphology.—The microörganism causing the disease actinomycosis is variously classified by different authors. Some look upon it as a streptothrix and class it with the so-called pleomorphous bacteria, others class it among the trichomycetes (hair fungi), and still others place it in a class by itself, intermediate between the bacteria (schizomycetes) and the lower moulds (trichomycetes). Quite a number of species of actinomyces exist (generally as saprophytes) in the outside world, and only occasionally invade the animal tissues, there to lead a parasitic existence.

Microscopic Examination of Fresh Material.—In making a microscopic examination for the actinomyces, or ray fungus, it is best to scrape some of the purulent material from an actinomycotic ulcer or abscess with a small scalpel and to spread this material on a slide, where it is covered with a cover-glass without making any pressure. If the preparation is now held against a dark background the so-called actinomyces granules can frequently be recognized with the naked eye. The majority are from 0.1 to 0.2 mm. in diameter and cannot be seen without magnification; but some are 0.5 mm. and more in size, and these appear as small yellowish, yellowish-green, or yellowish-brown granules to the naked eye. As these masses of fungi grow larger and older they generally appear more highly colored and more pronouncedly yellowish brown. If the preparation is now examined under the low power of the microscope the characteristic rosettes are seen, formed by club- or pear-shaped bodies arranged in a radiating manner around a common centre. From this typical arrangement, always shown in pus or tissues, the name actinomyces, or ray fungus, is derived. The rosette of clubs is frequently surrounded by lymphoid cells, polynuclear leukocytes, or giant cells. Sometimes

the fungi and the tissue cells surrounding them have undergone calcification, and the rosette appearance is then not so characteristic; it may, however, be brought out by the addition of dilute acetic acid, which will dissolve the lime salts.

Staining Properties.—It is unnecessary and in fact disadvantageous to attempt to prepare stained cover-glass preparations, because in preparing a thin spread the characteristic rosette form is destroyed. To study the finer structures of the actinomyces rosettes it is necessary to embed and section tissues containing them. The sections may be stained with hematoxylin and eosin, or, better still, by one of the following methods:

SCHLEGEL'S METHOD.—1. Stain celloidin sections for four to five hours in the incubator in a strong alcoholic solution of eosin—the alcohol-soluble eosin must be used, not the water soluble eosin.

2. Wash rapidly in 90 per cent. alcohol.
3. Stain for five to ten minutes in watery hematoxylin solution.
4. Wash in water.
5. Wash rapidly in alcohol.
6. Lift on slide with lifter.
7. Dry with filter paper.
8. Pour on some xylol or carbol-xylol to clear.
9. Mount in Canada balsam.

WEIGERT'S METHOD.—1. Stain sections well with borax-alum, or lithium carmin. Lithium carmin generally stains in twenty to thirty minutes; the other carmins must act for twenty-four hours.

2. Wash in acidulated alcohol (70 per cent. alcohol, 100 parts + HCl $\frac{1}{3}$ per cent.).
3. Wash in 95 per cent. alcohol.
4. Stain in anilin-water gentian violet for five to twenty minutes.
5. Wash in normal salt solution.
6. Gram's decolorizing solution a few seconds (iodin, 1 part; iodide of potash, 2 parts; water, 300 parts).
7. Decolorize in anilin-oil-xylol.
8. Wash in several changes of pure xylol.
9. Mount in Canada balsam.

MALLORY'S METHOD.—1. Stain sections for at least ten minutes in a saturated solution of water-soluble eosin.[1]

2. Wash rapidly in water.
3. Stain for a few minutes in anilin-water gentian violet.
4. Wash in normal salt solution.
5. Iodin solution (iodine, 1 part; iodide of potash, 2 parts; water, 100 parts).
6. Wash in water, lift on slide with a lifter, dry with filter paper.
7. Decolorize on slide with anilin oil, several changes.
8. Clear with xylol several changes.
9. Mount in Canada balsam.

[1] The section may also first be stained with alum carmin, but not very deeply.

Morphology of the Actinomyces Granule.—The last two methods given stain the filaments blue and the pear-shaped clubs yellow or pink. In sections stained by these methods the details of the structure of the rosette can be studied. The actinomyces granule in tissue or pus is found to be a more or less spherical or oval body which includes in its interior an only partially filled cavity. The wall of the cavity is composed of densely crowded, richly branched filaments, which on

FIG. 163

Actinomycosis of a fibrous cord of a horse. × 400. (Author's preparation.)

the whole have a radial direction. The interior of the cavity also contains branched filaments, but in comparison with the dense wall only in moderate numbers. Many of the filaments are quite slender and uniformly stained. They are provided with a delicate membrane. These filaments are not straight, but wavy; spirillum and spirocheta-shaped filaments are also present. The primary branches divide dichotomously. Lines of partition are seen in the filaments; they divide them into shorter segments, bacilla-like portions, and even

small, round, cocci-like bodies. The latter are the spores. They are not only found in the interior of the filaments, but also free between them. These, however, unlike the spores of the simple, true bacteria, have the same staining properties as the filaments, but they are more resistant than the filaments (see below). In addition to the slender well-stained filaments, there are seen coarse, poorly and irregularly stained threads, which are provided with a coarser, more densely stained membrane. Very pale threads containing stained round bodies, only here and there, are also seen. These are filaments in a well-advanced stage of degeneration. At the periphery of the actinomyces granule the free ends of the filaments have formed clubs, which are more or less round or more commonly elongated and truly club- or pear-shaped. These club-shaped formations are due to a *gelatinous degeneration* of the membrane of the coarser and less uniformly stained filaments; the latter can often be seen at the inner portion of the swollen club. The clubs are sometimes divided into segments by one or more lines of partition, and the individual parts forming them are often bulged out at the sides, so that the lines of division appear as constricting rings. All these morphologic features are due to degenerative changes of the membrane and of the inner protoplasmic thread of the filaments. The wall of the actinomyces granule or rosette is deficient in one place where the filaments partially filling the cavity project out of it into the surrounding pus or tissue. These projecting masses of filaments have been called the root of the actinomyces rosette. All of these features are well marked in comparatively young rosettes. When they grow older most of the details disappear and often nothing is left but a rosette formed by the degenerated gelatinous clubs. The best material for studying the finer details of the structure of the actinomyces granules is a soft granulomatous tissue, not a hard, fibrous one, which generally contains older actinomyces colonies which no longer show the finer details.

Cultural Properties.—All who have attempted to obtain actinomyces in pure cultures agree that this is a difficult task. Actinomycotic material must be obtained from the interior of a lesion in an aseptic manner and then numerous culture tubes must be inoculated, because in only a very small percentage will the fungus grow in a first generation. The material obtained is first rubbed up in a sterile mortar with the addition of melted gelatin or bouillon and then again inoculated into sterile melted gelatin tubes and their contents are finally poured into Petri dishes. Some of these must be kept under aërobic others under anaërobic conditions, because some varieties or species of actinomyces are aërobic, others anaërobic. When a first generation has once been obtained it is easy to secure the organism in a second and in subsequent generations on gelatin, glycerin agar, blood serum, potatoes, and in hen's and pigeon's eggs. On *gelatin plates*, if the fungus grows in a first generation under aërobic conditions, a small, gray point appears on the fifth or sixth day. Microscopic examination

shows it to consist of a fine network of filaments radiating from a common centre. After several more days the colony assumes a yellowish cloudy appearance. If one of these young first generation colonies is taken up with a strong platinum loop and rubbed over the surface of a *glycerin agar slant* and this is incubated, there appears, after twenty-four hours, on the surface in patches a thin, gray, moist, gelatinous film, which after another day becomes thicker and more cloudy. After a further lapse of time whitish points project above the surrounding surface and the growth, except at the very periphery, becomes opaque. In older colonies the former projecting points have become larger button-like masses which are elevated high over the surface of the culture medium. These masses now become yellow or yellowish red or even dark red (brick red), they are very firmly united with the culture medium soil by filaments penetrating into its depth, and it is difficult to remove them. They must be dislodged with a strong platinum loop or spatula. The condensation water of the culture tubes remains clear. In *gelatin stick culture* there is an abundant growth on the surface, but little along the stick-canal. The description here given refers particularly to actinomyces obtained by Bostroem from persons and domestic animals. The fungi are *facultative aërobes;* they grow much better in the presence of oxygen, but can also grow in the absence of atmospheric air. Wolf and Israel have, from two human cases, obtained actinomyces stems which grow very poorly under aërobic but very well under *anaërobic* conditions, particularly if inoculated into hen's or pigeon's eggs.

FIG. 164

Actinomycosis. Cover-glass preparation from pure culture showing true branching. × 1000. (Author's preparation.)

Morphology of the Fungus in Pure Culture.—If cover-glass preparations are made from pure cultures of actinomyces and stained with Loeffler's methylene blue or by Gram's method, slender and more coarse filaments which show true branching are seen. The filaments

are not stained uniformly, but they show unstained portions which are known as vacuoles. Some of the filaments are broken up into rod-like portions, others into round or oval granules. The latter are spores. They do not have the staining properties of the true bacterial spore, but are more resistant than the vegetative filaments. The characteristic rosettes seen in natural infection are never seen in artificial cultures.

Resistance.—Actinomyces cultures are quite resistant to drying out; they are killed, according to Domec, at 60° C. in five minutes, but the spores require 75° C. for five minutes before they are destroyed. According to others the spores can resist drying out for six years and are unaffected by 75° C. for fifteen minutes, but killed only by 80° C. acting for fifteen minutes.

Animal Inoculation.—On calves and small laboratory animals this is, as a rule, not successful. Sometimes a local self-limiting process occurs, but a progressing affection identical with the natural clinical course of the disease has perhaps never been produced.

Natural Infection.—It is now generally accepted that the disease is almost invariably transmitted to persons or susceptible animals through the hulls of grain, straw, hay, splinters of wood, etc., which are contaminated with the fungus and carry it deep down into the tissues. Johne's early observation in regard to finding actinomyces-bearing barley hulls in the tonsils of swine has already been referred to. Since then numerous observers have succeeded in finding such infected material in the tissues of actinomycotic lesions in man and animals. Bostroem examined 32 cases of actinomycosis of the upper or lower jaw in cattle, and he regularly found hulls deeply wedged in between the teeth and the gums, or he found them still deeper in the osseous granulations. These hulls were studded with ray fungi. Bang showed that the ray fungus grows well on grains, particularly on barley. Berestnew succeeded in finding ray fungi on dry grasses, grains, and straw by introducing them into sand kept in glass vessels in the incubator and moistening the vegetable parts with sterile water. Under these conditions colonies of ray fungi developed on the material collected in the outside world. The mode of transmission through infected grain has also been shown to be true of man in whom it has been repeatedly demonstrated that the entrance of hulls into the tissues of the mouth, the pharynx, the hands, etc., has been followed directly by the development of actinomycotic lesions. There is no convincing evidence of direct transmission from one sick animal to another, and no case has ever been reported to show contagion from cattle or other animals to man.

Frequency of Natural Infection.—In man actinomycosis is comparatively rare; in cattle it is very common and occurs among them both sporadically and endemically, and occasionally as an epidemic. In certain localities the fungus is evidently widespread and the opportunities for infection are numerous.

The spreading in the tissues along the lymphatics occurs probably through the filaments which protrude from the rosette cavity at its root and penetrate into the surrounding tissues, in which small bacilli-like segments and spores become detached. They are taken up by the leukocytes, which later wander away and, instead of killing the fungi by phagocytic action, perish themselves in consequence of cell necrosis. The spores set free in this manner form the focus for the formation of new actinomycotic granules or rosettes. The spreading of the infection by continuity depends to a large degree upon the tissue reaction. Where much fibrous connective tissue is formed around the actinomyces granules, they are likely to undergo complete gelatinous degeneration with club formation, and to die out. Where there is more tissue necrosis and softening and less fiber formation the ray fungi tend to remain alive, to form new young filaments, and to spread through these extending filaments and their rods and spores.

Actinobacillosis.—Lignière and Spitz, in 1902, described a disease of cattle in Argentina, which is said to occur sporadically and also in epizoötic form. This disease was called actinobacillosis. Its symptoms and pathologic changes are almost identical with those of actinomycosis in cattle. The lesions are most commonly found in the subcutaneous tissue of the neck; the tongue, however, has been found affected in only 5 per cent. of the cases; changes in the lungs, lymph glands, lymphatics, pharynx, udder, and bones were found even less frequently. The subcutaneous changes consist in diffuse infiltrations with abscess formation which project as round swellings above the surrounding surface. The tumors are generally not very large but of rather moderate size, and only after having existed for weeks and months do they discharge a very tenacious whitish or greenish pus, in which grayish-white granules of the size of a pinhead can be seen. The cavities of the abscesses contain a grayish granulation tissue. The disease, however, more frequently leads to the production of hard, fibrous connective-tissue masses, rather than causing abscess and granulation tissue formation. When the process invades the tongue the picture of the *wooden tongue* of ordinary actinomycosis is produced. Microscopic examination of the granules found in pus shows them to resemble the rosettes of ordinary actinomycosis. Lignière and Spitz, however, claim that if the granules are rubbed up in a mortar and inoculated into culture media, a growth of bacilli not much larger than 1 to 1.8 micron long and 0.4 wide is formed. Inoculations of these organisms into cattle are said to have produced lesions like those found under natural conditions.

QUESTIONS.

1. What is the technical name for lumpy jaw in cattle? What organism causes it?
2. For what pathologic process was the disease formerly generally mistaken, and why?
3. Who were the investigators who first saw the ray fungus? Who first rceognized its significance?
4. Where does the ray fungus exist in the outside world?
5. To what changes does it lead after invading the tissues of susceptible animals?
6. Describe the actinomycotic tongue affections in cattle. Why called under some circumstances the wooden tongue?
7. Describe the spread of the disease in the maxillary bones; also the final appearance of an advanced case.
8. What is an osteoplastic inflammation?
9. What is meant by necrosis of the bone?
10. Which internal organ in cattle is frequently the seat of actinomycotic lesions in advanced cases?
11. What animals besides cattle are susceptible to actinomycosis?
12. What lesions does actinomycosis produce in man?
13. Discuss the systematic classification of the ray fungus.
14. Describe the process for the microscopic examination of actinomycotic granulation tissue or pus.
15. Describe the ray fungus as generally seen in pus.
16. Describe some methods of staining the ray fungus in celloidin sections.
17. Describe the finer details of an actinomyces granule as it can be studied in a section.
18. What are the clubs of the ray fungus which form the rosette?
19. How is a first culture from actinomycotic material obtained?
20. Describe the cultural properties of the ray fungus.
21. Describe the appearance of the ray fungus in a stained cover-glass preparation obtained from a pure culture.
22. Discuss the resistance of the ray fungus.
23. What animals are very susceptible to artificial ray fungus inoculation?
24. How is actinomycosis generally contracted by cattle, horses, hogs?
25. How is it generally transferred from cattle to man?
26. How can the presence of actinomycosis on grasses be sometimes demonstrated?
27. How does the ray fungus spread after it has once penetrated into the tissues?

CHAPTER XXXVIII.

HIGHER HYPHOMYCETES AS THE CAUSE OF DISEASE—LEECHES, OR BURSATTEE—PNEUMONOMYCOSIS—DERMATOMYCOSIS—TRICHOPHYTON TONSURANS—ACHORION SCHÖNLEINII—FUSARIUM EQUINORUM—OIDIUM ALBICANS.

In the preceding two chapters the diseases due to trichomycetes, including streptothrix and actinomyces infections, have been considered. These organisms belong to the lower hyphomycetes, but the higher hyphomycetes are likewise the cause of some animal diseases.

LEECHES, OR BURSATTEE.

Under the name of leeches, bursattee, summer sore, hyphomykosis destruens equi, "Bösartige Schimmel-Krankheit der Pferde" (German), a disease of horses apparently due to hyphomycetes has been described. Whether the disease found in India and other parts of Asia is absolutely identical with the similar affection reported in the United States is a question not fully settled. The name bursattee is derived from the Indian word *burus*, rain, because it was believed that some causal relation existed between the disease and the rainy season in India. The disease in India has been described by F. Smith and Steel and in the Sunda Islands by DeHaan and Hoogkamer. The pathologic lesions there noticed consist in very firm nodules below the skin, particularly in the lips, the alæ of the nose, the eyelids, but also on the body and the extremities and in the mucosa of the mouth and nasal cavities. The nodules in the mucosa later change into open ulcers covered by an easily bleeding granulation tissue surrounded by ragged, uneven margins. The ulcers burrow deep down in the tissue, invade the bone, sometimes perforate it, and form fistulous tracts. In the latter and in the granulation tissue grayish-yellow masses or plugs are found, firmly adherent to the surrounding tissue and sometimes calcified. In these masses the *mycelia* and *spores* of a mould are found, which was obtained by DeHaan in pure culture. Inoculation experiments, however, failed to reproduce the disease. As it occurs in the United States the disease was first more fully described by Neal, of Florida, who noticed it in equines and also in cattle which had spent much time in ponds and swamps. Dawson, of Florida, has stated that leeches, or bursattee, in Florida is a common disease characterized by the formation of tumor-like masses with

some of the features of actinomycosis. In the granulation tissue yellow bodies with root-like projections are found, called leeches by the natives. These bodies consist of the mycelium of the fungus. Dawson saw the disease in horses only, not in cattle. Fish examined tissues containing the fungus and described the latter; he did not, however, obtain it in pure cultures. The mould is seen in the tissues in the form of filaments which sometimes have club-shaped ends and some of which show septa. The mycelium is branched and small, round bodies; evidently spores are found in it. The mycelium is often embedded in a calcified matrix which is formed in consequence of the inflammatory reaction of the tissue.

PNEUMONOMYCOSIS.

Infection of the lungs of man and animals by hyphomycetes or moulds is comparatively rare, but a sufficient number of cases have been reported to show that such infections do undoubtedly occur. In animals they are generally observed among those kept in warm, moist, poorly ventilated places and fed on mouldy feed. Such mould infections of the lungs are most frequently seen in domestic birds; pigeons appear to be most susceptible, but chickens, ducks, and geese, as well as parrots and birds in zoölogical gardens, may also be infected. The disease is less frequently found in mammals. Schütz, Rivolta, Martin, Bollinger, Lucet, and Peck have reported cases in horses. Roeckl, Piana, Bournay, Ravenel, and Hartenstein in cattle; others in sheep, deer, and dogs.

The pathologic changes produced by the inhaled and multiplying moulds consist in the production of dirty yellow, greenish, mouldy looking patches, which lead to ulceration, with plugging up of the lumen of bronchioles and the formation of catarrhal foci in the pulmonary parenchyma. These may be purulent, caseous, or mortar-like in character; sometimes they are surrounded by a fibrous capsule or infiltrate the neighboring tissue in a diffuse manner. The moulds causing these changes are found in the patches and in the interior of the pathologic foci. Microscopic examination is always necessary, as some of the mycotic lesions closely resemble tubercular changes and in horses they have resembled glanders.

The moulds which have been found in cases of pneumonomycosis are the following: *Aspergillus fumigatus* and *Aspergillus nigrescens* form a colorless mycelium from which straight, unbranched fruit bearers arise. On their upper ends the fruit bearers carry a columella and a sporangium which produces numerous spores (conidia) arranged in radial rows. On *bread*, Aspergillus fumigatus forms a bluish-green and later ashy-gray mould; Aspergillus nigrescens forms a blackish- or chocolate-brown growth. Aspergillus fumigatus is the mould most commonly found in mycosis in birds. Birds can easily be infected

experimentally by keeping them for a short time in a closed space where the air contains spores of this mould. Other moulds which have occasionally been found as the cause of pneumonomycosis and other internal affections are *Aspergillus flavus, niger,* and *subfucus; Mucor rhizopodiformis* and *corymbifer.* All of these when injected intravenously into rabbits in an emulsion containing numerous spores, produce multiple mycotic foci in the internal organs from which the animals die. Natural infection with them are, however, rare.

DERMATOMYCOSES.

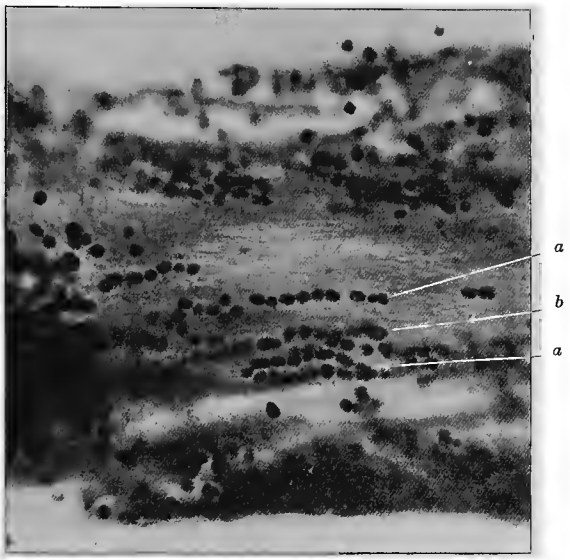
A variety of diseases of the skin due to hyphomycetes or moulds occur in man and the domestic animals. The most important of the microörganisms which cause such affections, known under the collective name of dermatomycoses, are the following:

Trichophyton Tonsurans.—This mould is the cause of the skin disease known as herpes tonsurans, characterized by the formation of scales and the falling out of the hair. It has been found associated with this affection in man, the horse, dog, cat, goat, sheep, and hog. It was discovered by Gruby (1842) and Malmsten (1846).

Method of Examination.—In order to examine this and other moulds causing various dermatomycoses, some scales with attached hairs must be removed from the skin by scraping with a scalpel. The material is then best placed in a test-tube and well shaken for some time with chloroform, in order to extract the fat. After this has been accomplished, the chloroform is poured off and its last remnants are allowed to evaporate from the scales and hairs. The latter are then placed on a slide soaked in a $33\frac{1}{3}$ per cent. solution of caustic soda or potash, covered with a cover-glass and examined in this fluid under a higher power dry lens of the microscope. The scales and hairs are immersed in the strong alkaline solution in order to make them transparent so that the filaments and the spores of the mould may become readily visible. They are then seen surrounding the hair as a septate mycelium and as rows of highly refractive round bodies which are the spores. So many of the latter are usually present that it is often impossible to detect the filaments; they can, however, generally be more easily seen in the scales than in the hairs. The spores, as a rule, measure 4 to 6 micra in diameter, but they may vary between 2 to 8 micra. The hyphen or filaments are about 4 micra thick. Pure cultures are difficult to obtain, since the hair and scales of skin also contain numerous bacteria. Sabouraud has succeeded in getting a growth of the Trichophyton tonsurans in a beerwort medium composed of maltose 4 parts, peptone 2 parts, tinctura fucus crispa 1.5 parts, and water to make 100 parts. Kral's method consists in rubbing up the hairs with sterile silicon powder and inoculating it in gelatin tubes, from which plates are poured. Kitt succeeded in

washing the scales and hairs in an alkaline (potassium hydroxide) solution, which killed the bacteria, but left enough mould intact for subsequent successful inoculations. The trichophyton grows best at

FIG. 165



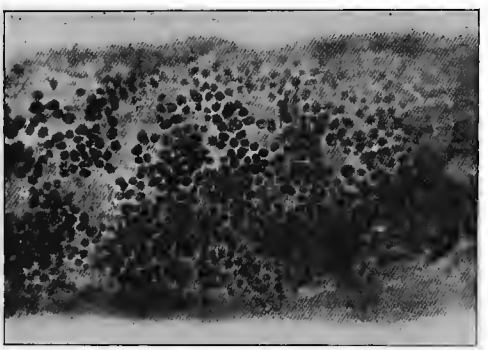
Portion of a hair invaded by the Trichophyton endo-ectothrix. × 550. *a*, *a*, chains of spores in focus; *b*. a chain situated farther within the hair, and hence not in focus. (From a photomicrograph.)

FIG. 166

Trichophyton ectothrix culture, three weeks old, from a case of Tinea sycosis. (Mewborn.)

about 30° C. It liquefies *gelatin*, on which it forms a white, dusty cover which is difficult to break up. On *potatoes* a folded felty, sometimes white, at other times yellowish, reddish, or dark membrane is formed. If examined microscopically the growth shows a septate, colorless mycelium, and round or oval chlamydospores. Different cultures vary considerably in certain features, so that a number of varieties have been distinguished, such as Trichophyton megalosporon (large spores), Trichophyton microsporon (small spores), and several others. Spores in pure cultures are generally formed within the hyphen as chlamydospores. In the skin and hairs they are formed by the breaking up of the filaments into segments (gemmæ).

FIG. 167



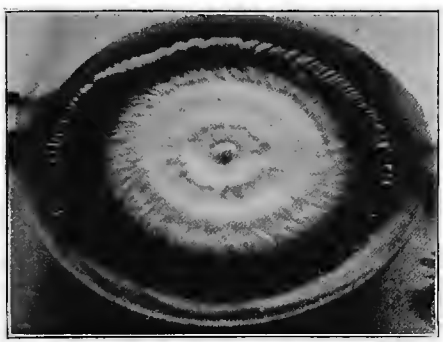
Portion of a hair showing the Microsporon Audouini. (From a photomicrograph.)

One of the varieties of the Trychophyton tonsurans has been called Microsporon Audouini, and two of its subvarieties, the equinum and the caninum, have been found, respectively, in horses and dogs. Certain authors, however, claim that the different varieties of Trichophyton tonsurans are really a single species producing practically the same infection, only somewhat modified as to the species of animal infected. The mode of infection consists in that the mould first penetrate into the hair roots, where they multiply and surround the hair with a complete mantle and then enter into its interior. Trichophyton tonsurans infections have also been observed a few times in domestic birds.

Achorion Schönleinii.—This mould is the cause of the disease known as favus, dermatomycosis achorina, tinea favosa, "Wabengrind" or "Erbgrind der Säugethiere" (German). It occurs in man, mice, rats, cats, dogs, and rabbits. A few cases which were described as appearing in horses and cattle were probably trichophyton infections. Favus infection is characterized by the formation of scaly crusts, which are depressed in the centre and sulphur yellow, at least in the interior, where the color has not been changed by external influences.

The mould is found in the scales of the skin, in the form of homogeneous or granular septate and branched hyphens, 3 to 5 micra thick. The filaments are often thinned out at their free ends and thickened at the points where branches arise. Some of the hyphens

FIG. 168



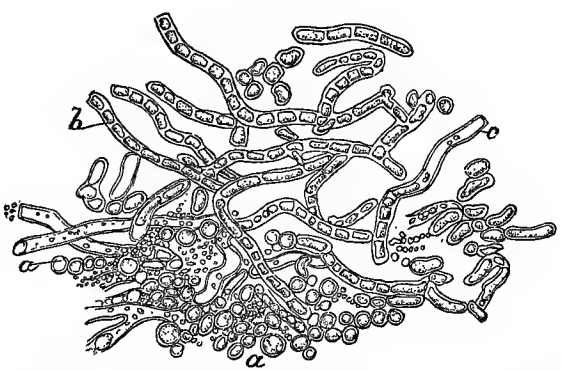
Culture three weeks old from ringworm of cat contracted from ringworm of girl's face. (Mewborn.)

FIG. 169

Culture of Achorion Schönleinii. (Mewborn.)

are broken up into oval spores 3 to 6 micra in diameter. Sometimes the mould is found in the skin as a very dense, felted mycelium. Achorion Schönleinii requires more proteid material for its growth on artificial media than Trichophyton tonsurans. Its optimum temper-

FIG. 170



Achorion Schönleinii: *a*, spores; *b*, *c*, sporophores. (After Cornil and Ranvier.)

ature is at 25° C. On gelatin its growth somewhat resembles trichophyton, but the medium is only liquefied after several weeks. On *potatoes* and *beets* an elevated, folded, grayish-white growth is formed, which later becomes grayish yellow. It grows well on agar kept in

the incubator. Artificial cultures of this mould exhibit considerable pleomorphism, and this has led to the establishment of several varieties. Achorion infects younger persons or animals more easily than older ones. Mice are very frequently infected, and these, when caught by cats, infect the latter. Man is infected frequently from animals.

Achorion Keratophagus.—This organism was named and described by Ercolani. It is of the achorion type and was alleged to have been the cause of an onychomycosis of donkeys and mules. The affection is characterized by the formation of cavities between the hoof and the lamina sensitiva and the invasion of the former by the mould. It has not been possible to produce the disease artificially by inoculation.

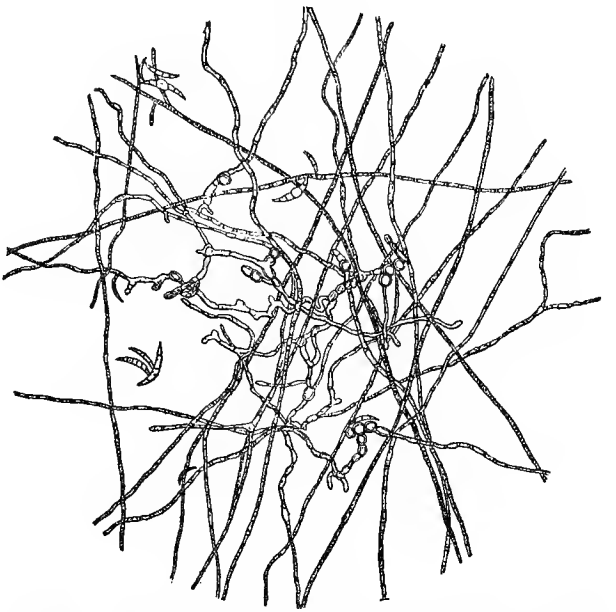
Lopophyton Gallinarum.—This organism, also known as Dermatomyces or Epidermophyton gallinarum, is a variety of Achorion Schönleinii and causes favus or tinea cristæ galli in chickens. The organism affects the comb and forms on it whitish, mouldy looking spots which finally cover the entire comb with a white layer which increases in thickness until it finally forms a crust up to 8 mm. thick. Two types of hyphens are found in the lesions, those of the one are long and wavy, 2 to 5 micra thick, with irregular side branches; the others are short, straight, or curved filaments, sometimes dichotomously divided, composed of three to four thick-walled segments, which contain a highly refractive protoplasm. The hyphens of this type are from 4 to 6 micra thick and later fall apart. Some investigators have looked upon the segments as spores. Cultures of this variety of achorion found on chickens grow slowly and form an intensely red pigment; they liquefy gelatin.

Fusarium Equinum.—This name was given by Noergaard to a fusarium[1] found by Melvin and Mohler as the apparent cause of a dermatomycosis in many hundred horses in the Umatilla Indian Reservation in Oregon. Fusaria are hyphomycetes with septate mycelia (mycomycetes) and belong to the class of ascomycetes. These are moulds which develop an ascus or sac in which endogenous spores are formed. The spores formed in such a special spore sac or ascus are known as ascospores. This organism, it appears, enters the hair follicles, where it multiplies, bringing about an irritation which causes pruritus and the formation of a little scurf around two or three hairs. When the scurf is rubbed off a red, moist, denuded surface is left in its place. The spores of the organism can be seen in sections of the skin. They are especially numerous in the hair follicles; mycelial threads are likewise encountered. Melvin and Mohler succeeded in obtaining the organism in pure cultures and found that the growth remained pure white on all media except plain *Dunham's solution* on which the lower surface in contact with the fluid became a chocolate

[1] It is described fully in the Twenty-fourth Annual Report of the Bureau of Animal Industry, 1907, Dermal Mycosis, etc., Melvin and Mohler, p. 260.

color. In *agar* the growth first assumes a salmon-pink color, but later becomes white. *Potato* is a very favorable medium for the organism. The frost-like film which is formed in three to four days on culture

FIG. 171



Conidia and mycelium of Fusarium equinum cultivated from root of hair of horse affected with dermal mycosis. (Melvin and Mohler.)

FIG. 172

Photomicrograph of a five-day-old colony of the fusarium on an agar plate. (Melvin and Mohler.)

media is composed of septate branching hyphens, or filaments. The organism forms microconidia, macroconidia, and chlamydo spores. The large falcate, crescentic, or sickle-shaped macroconidia or spores, from 25 to 55 micra long and $2\frac{1}{2}$ to $4\frac{1}{2}$ micra wide, are typical for all fusaria; they commence to germinate in form three to ten hours after being transplanted into a favorable new culture medium. From a peculiar skin lesion in a hog, Hart isolated a culture of fusarium which seems to be identical with the above Fusarium equinum. Peters found Fusarium moniliforme, discovered by Sheldon, on corn as the cause of a disease of horses in which they lose their hair and hoofs. The same organism is also said to have caused loss of hair in cattle and hogs and of feathers in chickens.

Oidium Albicans.—The disease known as thrush, soor, or stomatitis oidica is an inflammation of the mucous membrane of the mouth and pharynx. It occurs in children, domestic birds, and also calves and other young domestic mammals.

The pathologic lesions consist in the formation of grayish-white points and smaller or larger dots or even quite extensive pseudomembranes. The lesions later become brownish. If the pseudomembranes are removed a slightly reddened but otherwise unchanged mucous membrane is seen. The pseudomembranes are, however, quite firmly adherent. If examined microscopically they are found to consist of desquamated swollen epithelia mixed with numerous wavy or straight filaments and oval bodies. These formations are the mycelium and spores of the fungus which causes the disease and which is known as Oidium albicans or Monilia candida. The fungus is widespread in air, water, and on decaying vegetable matter as a saprophyte, and only occasionally infects the buccal and pharyngeal membranes of young beings. The mycelia are composed of cylindrical cells, 1 to 4 micra wide and 10 to 20 micra long. The filaments show branching and the outer ends are rounded off or club-shaped. The clubs often contain oval, highly refractive bodies, the *gonidia,* or *spores,* which are also found free between the filaments. The free spores in air and food come in contact with the buccal or pharyngeal mucosa, and if there are slight epithelial defects the gonidia may develop and lead to the formation of thrush spots and membranes. Cases have also been observed in children in which the fungus had penetrated deeper and led to metastases in the internal organs. The pseudomembranes are best examined in very dilute acetic acid (1 to 3 per cent.), which brings out the mycelia and spores under the microscope.

The organism can be easily cultivated in artificial cultures. It is best to use distinctly acid media, because bacteria will not easily develop on them, but Oidium albicans does. *Fresh disks of apples,* obtained after washing the apples externally and dividing them with a sterile knife, form a good culture medium for this fungus. It also grows on *agar, gelatin, coagulated blood serum,* and *potatoes.* The

colonies on gelatin plates are milk white, on potatoes yellowish or grayish white, and give off an odor of sour beer. The growth in *gelatin stick cultures* shows the nail form; the medium is not liquefied. The development on all these media occurs at room temperature, more rapidly in the incubator. The organism on media containing sugar assumes a yeast-cell type, while on media which contains no sugar a mycelium is formed. If the organism is inoculated into the crops of pigeons the typical thrush lesions are produced. If injected intravenously into rabbits multiple metastatic foci of infection are produced and the animals die. Young pigeons can be inoculated in the mouth, where thrush lesions are formed. Older pigeons and chickens are generally resistant.

In artificial cultures Oidium albicans and other oidia often appear as individual cells and multiply by budding; hence they are evidently closely related to the budding fungi, or blastomycetes, discussed in the next chapter.

QUESTIONS

1. What is leeches or bursattee? In what countries encountered?
2. What are the pathologic lesions of the disease?
3. Describe the mould found in the pathologic changes.
4. What is meant by pneumonomycosis? In what animals does it occur and under what conditions?
5. What moulds cause pneumonomycosis?
6. What is meant by dermatomycosis?
7. What mould causes herpes tonsurans?
8. Describe the lesions in herpes tonsurans and the method of finding the mould causing it.
9. How does it look under the microscope?
10. Describe pure cultures of Trichophyton tonsurans and the methods of obtaining them.
11. In what animal does the Microsporon Audouini cause skin disease?
12. What are the lesions of favus? What organism causes this skin affection?
13. Describe the favus mould.
14. Describe its cultural properties.
15. What lesions are caused by Achorion keratophagus?
16. What is Lopophyton gallinarum? Describe the lesions it produces.
17. What is the Fusarium equinum? Describe the lesions it produces.
18. What variety of spores does the organism form? What type is characteristic for the genus fusarium?
19. Describe the morphologic and cultural properties of the organism.
20. Where has Fusarium moniliforme been found?
21. What organism causes thrush?
22. Where and in what animals is this affection found?
23. What lesions does it produce?
24. What is the other name for Oidium albicans?
25. Describe its morphology.
26. How will you look for it in the lesions of thrush?
27. Describe the cultural properties of Oidium albicans.
28. To what organisms is oidium related?

CHAPTER XXXIX.

BLASTOMYCES—EPIZOÖTIC LYMPHANGITIS IN HORSES—BLASTOMYCOTIC DERMATITIS.

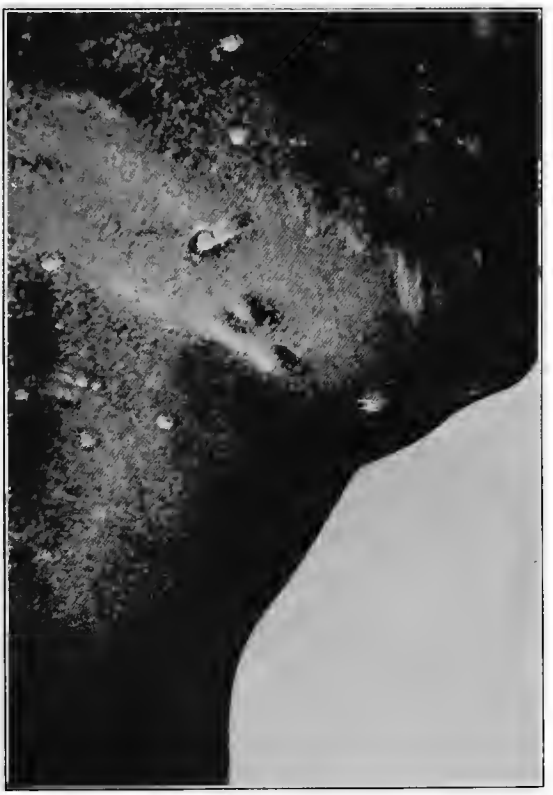
BLASTOMYCES.

THERE is a large class of low vegetable microörganisms of which the common brewer's and baker's yeast are the best-known types. A few of these have been found as the cause of disease in man and the lower animals. The classification of these fungi has been very difficult, and botanically they have been defined as ascomycetes, because, like these, they generally form a number of endogenous spores in a *spore sac* or *ascus*. Yeast cells, however, frequently present themselves as simple, more or less globular, strictly unicellular organisms, and in them the whole cell becomes the ascus, or spore sac, in which the *endogenous* ascospores are formed. As Lafar has pointed out, the term *saccharomyces* (often improperly used in pathology) should be reserved for the sugar-fermenting organisms of this type, since the word means sugar fungi. On the other hand, certain organisms of this class have never been known to form spores, and they cannot consistently be classified as ascomycetes. It appears, however, that they have the common property of *forming buds, i. e.*, one or more nipple- or sac-like or globular offshoots or out-growths, which later become separated from the main portion by a partition wall or septum, drop off and form new independent unicellular organisms. This process of multiplication is known as *budding*, hence the organisms which show this mode of multiplication are known as *blastomycetes*, or budding fungi. Most of the latter can also multiply by sporulation, because they form more than one spore—up to eight and higher. Those blastomycetes which do not form spores are grouped under the genus *Torula*.

When blastomycetes are budding the newly formed buds are not always necessarily cut off, so that the unicellular type is preserved. Under some conditions both the mother cell and the bud elongate, become elliptical or cylindrical, and adhere together. The process is repeated a number of times at the outer ends of the connected cells, and there are formed in this manner filaments and, indeed, a structure with branching effects much like the mycelia of the hyphomycetes. It has been ascertained that the saccharomycetes and probably the blastomycetes in general possess a nucleus with a nuclear membrane and nucleolus. These structures can only be seen exceptionally in

unstained specimens, and even in stained specimens, they are exhibited with difficulty; in young, resting cells, and by special staining methods. To exhibit the nuclear structures, the yeast cells must first be fixed in picric acid, and after having washed this out they must be stained with Haidenhain's iron-hematoxylin. It has also been shown that the nucleus of the yeast cell divides by a simple *karyokinetic* or by an *amitotic type* when a bud is produced or when the endogenous asco-

FIG. 173



Pseudofarcy, or blastomycosis, in a Filippino pony. The picture shows several swollen, subcutaneous lymph nodes and one open ulcer. (Strong.)

spores are formed. The latter are generally somewhat more resistant, particularly to drying out, than the adult vegetative organism. In addition to the nucleus the vegetative adult organism also often exhibits much more readily seen vacuoles filled with fluid and in old cells highly refractive granules, which consist of proteid material, but also contain some fat. In cover-glass preparations the blastomyces can be stained by the ordinary watery anilin stains; in tissues they are best

exhibited by the eosin-alkaline methylene-blue method of Mallory. In *artificial cultures* they often form thick membranes or pellicles on the surface of the culture medium and some of them form zoögleal masses.

PSEUDOFARCY, OR EPIZOÖTIC LYMPHANGITIS.

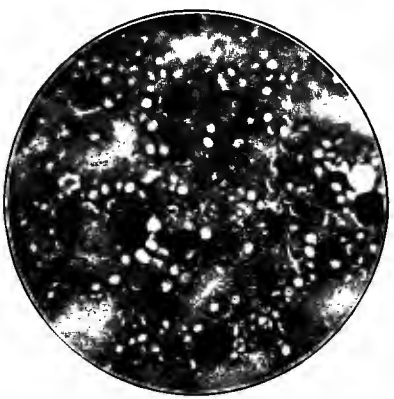
Occurrence and Pathologic Lesions.—Under the above names a disease of horses, which is also known as saccharomycosis, or blastomycosis farciminosus, has been described. Clinically it resembles the cutaneous type of glanders, or farcy, and has therefore been called pseudofarcy. It was first described by Italian and French observers; later it was reported from Japan and other Asiatic countries, including the Philippine Islands, where it was found by Strong. A few cases have also been encountered in the United States. The pathologic changes present themselves as hypertrophies of the subcutaneous connective tissue, particularly along the lymphatics. The tissue increase leads to the formation of distinct nodules. If these are incised they generally discharge a thick pus or coagulated lymph, which, under the microscope, shows yeast-cell-like bodies both within and without the tissue cells. The lymph glands in the neighborhood of the nodules are swollen; they sometimes contain purulent foci which show the parasites. The latter may also form metastatic foci in the internal organs. According to Tokishige the disease has also been observed in cattle in Japan.

Morphology.—The parasite found in the purulent and necrotic foci was first seen by Rivolta and named *Cryptococcus farciminosus*. As later observations have shown it belongs to the budding fungi. The organisms show a double contoured membrane; they are round or oval, and measure from 2 to 4 micra in length and 2.5 to 3.6 micra in width. The poles of the oval bodies are generally somewhat pointed, sometimes buds are seen on the cells which are found in the pathologic product. The contents of the parasitic cells are sometimes perfectly homogeneous; at other times they show a small coccus-like nucleus (0.5 to 1 micron in diameter), or contain in their interior rather coarse protoplasmic granules. Tissue cells often are full of the parasites, and a number of them may also be seen in leukocytes. The pus also contains shrunken, irregular, or crescentic blastomyces. The organisms stain with the ordinary watery anilin stains, and are Gram positive.

Cultural Properties.—Tokishige and others have obtained pure cultures of these blastomyces. They grow in agar, gelatin, bouillon, on potatoes, and in other media, but the development is very slow. The colonies may be visible after ten days, or it may take thirty days before they appear. On *agar* they form grayish-white elevated granules from 1 to 4 mm. in diameter, which become somewhat confluent, and form worm-like or intestine-like conglomerations. The

growth is comparatively compact and difficult to remove with the platinum loop. On *gelatin*, after several weeks, a yellowish, sand-like mass is formed, liquefaction of the medium does not occur. On *potatoes* the growth appears more rapidly, and is of a dirty white, smooth, lusterless character. In *bouillon*, whitish flocculi, which slowly sink to the bottom, are formed. Inoculation of pure cultures into horses and small laboratory animals is rarely if ever followed by the production of typical lesions, but pus containing the organisms when inoculated into equines has produced the picture of the disease. Tokishige was able to infect horses and to produce abscesses and nodules. None of the experimental inoculations brought about a progressive fatal case. Strong produced nodules in monkeys inoculated with pus from naturally infected horses. As the organism in artificial cultures does not ferment any of the sugars, the names Saccharomyces farciminosus and Lymphangitis saccharomycotica are misnomers and should be replaced by Blastomyces farciminosus and Lymphangitis blastomycotica. The organism in artificial cultures forms mycelia-like filaments composed of oblong or cylindrical cells.

FIG. 174



Photomicrograph of pus from a lymph nodule in a horse suffering from blastomycosis, showing intra- and extra-cellular blastomyces. (Strong.)

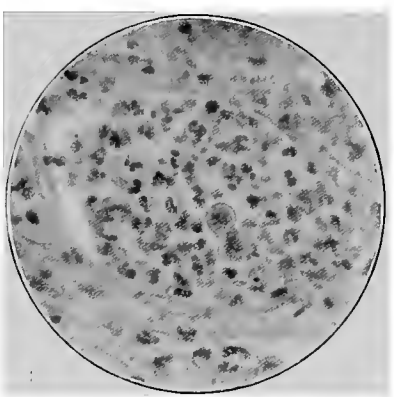
FIG. 175

Blastomycotic lymphangitis in a North Dakota mare. (Mohler.)

A TORULA AS THE CAUSE OF A TUMOR IN A HORSE.

Frothingham has described a tumor-like lesion in the lung of a horse caused by a blastomyces. It was situated in the posterior portion of the caudal lobe of the right lung, and was about again as large as a human head. In smears and sections a great number of blastomycetes were seen. These were obtained in pure cultures on *potatoes* and *other media*. On *gelatin plates* the organism formed, in five to seven days, white, elevated, pinhead colonies. On *potatoes* the growth was at first white, soon becoming a dirty gray, and after a few days gradually taking on a chocolate-brown color. In old cultures the growth upon the less nutritive portions of the medium becomes white and dry, and resembles lime deposits. The color varies quite widely; sometimes it remains a lighter or darker yellow, at other times it assumes the deep brown color almost immediately. The organisms are slightly oval and vary greatly in size. The young forms are surrounded by a delicate membrane which in older forms becomes much thicker. The cells in cultures are sometimes included in a gelatinous matrix. As the organism during a long observation, did not produce spores nor ferment dextrose, lactose, or saccharose, it was classified as a torula.

FIG. 176



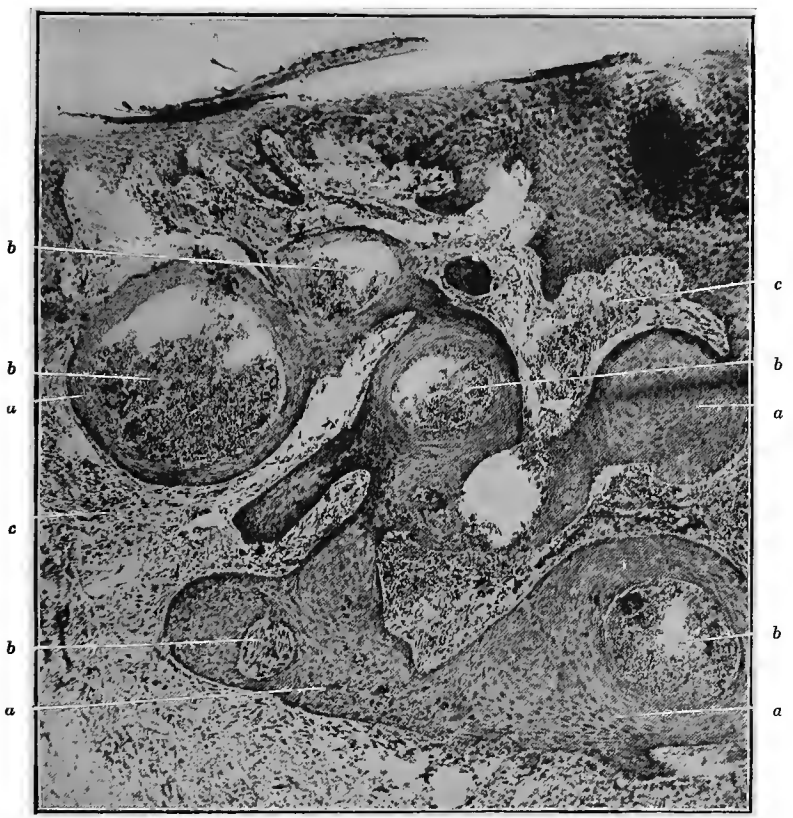
Section through the skin in a case of blastomycotic dermatitis in man, showing small abscess cavity in the epithelial layers, near the centre a budding parasite. × 1000. (Author's preparation.)

BLASTOMYCOTIC DERMATITIS IN MAN.

A disease of man, particularly observed in the United States, and known as blastomycotic dermatitis or cutaneous blastomycosis, is due to an organism or a variety of organisms of the type under discussion. Cases of this kind have been reported by Gilchrist, Hektoen,

LeCount, Montgomery and Hyde, Ricketts, Anthony and Herzog, and others. Clinically they closely resemble certain skin cancers or the warty form of skin tuberculosis (tuberculosis verrucosa cutis), and they were formerly mistaken for these affections. The histologic features of blastomycotic dermatitis are a hypertrophy of the epithelial layers with the formation of pegs and bands, as seen in carcinoma, and an inflammatory reaction in the derma and the subcutaneous

FIG. 177



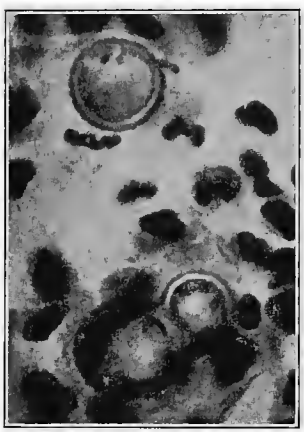
Blastomycosis of the skin. Vertical section from a typical lesion. *a*, hyperplasia of rete; *b*, abscesses in epithelium; *c*, infiltration of cutis. × 55.

connective tissue. In the hypertrophied masses of epithelial cells *miliary abscesses* are found which contain the *budding fungi*. These can best be demonstrated in sections by the eosin-methylene-blue stain, and they can also be seen in squeezed-out pus in the unstained, moist cover-glass preparation. Hyde and Montgomery have described pure cultures of these organisms raised on *glycerin* and *glucose agar* as follows:

The time required for the development of the different organisms

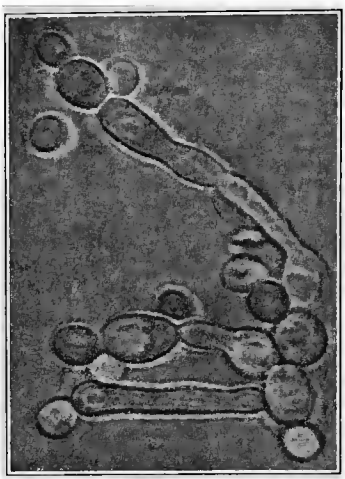
in the original cultures varies from two to sixteen days, the majority showing a growth in from two to eight days. Subcultures appear in from two to five days. In gross appearances the cultures may show slightly elevated, white, smooth colonies or irregular areas following the track of the needle; a translucent, gelatinous or yellowish-brown and pasty growth; a roughly granular surface, which may eventually form prominent folds and depressions; a light, white (in older cultures slightly yellow or yellowish brown) fluffy growth, with short or long aerial hyphæ; or a central white, elevated portion, which may be fluffy or covered with short projections like white hairs, and which is surrounded by a translucent, non-elevated zone. With very few exceptions, the growth extends more or less into the medium and becomes closely incorporated with it.

FIG. 178



Blastomycosis of the skin. Budding organism in tissue. × 1200.

FIG. 179



Blastomycosis of the skin. Hanging drop. × 1200.

Moist preparations from the cultures may show budding organisms; or fine, homogeneous, and branching mycelia, more or less segmented, with or without lateral conidia, which may contain few or many highly refractive bodies, varying in size. The latter are probably spores, though in some instances they may be oil drops. Mingled with the mycelium may be seen round, oval, or irregular double-contoured bodies, varying greatly in size, and more or less filled with highly refractive globular bodies.

The organism in a number of the reported cases has formed multiple metastatic abscesses through the general circulation and lead to death. The blastomycotic infection in man as the similar infection in the horse in pseudofarcy is, therefore, a dangerous disease.

BLASTOMYCES THE CAUSE OF TUMORS?

A number of investigators, particularly Sanfelice, have claimed that blastomyces are the cause of malignant tumors in man and the lower animals, but these claims have not been confirmed. Sanfelice reported the finding of a pathogenic blastomyces in a cancer of the liver in an ox, and since the pathologic lesion was calcified he named the organism *Saccharomyces lithogenes* (lithogenes, stone-forming). Another organism obtained from nodules in the lung of a hog was described by the same author as *Saccharomyces granulomatogenes.*[1]

QUESTIONS.

1. What is the common English name for saccharomyces?
2. What is the meaning of the term blastomyces?
3. Describe the process of budding.
4. Why have saccharomycetes been classified as ascomycetes?
5. Define the terms: Ascus, endogenous ascospares.
6. What is the difference between a typical saccharomyces and a torula?
7. Do saccharomycetes, by budding, always form new individual unicellular globular organisms? If not, what do they form?
8. Describe the finer details of the structure of a blastomyces.
9. How can blastomyces be stained in cover-glass preparations? How in tissue sections?
10. What is pseudofarcy in horses?
11. Describe its pathologic lesions.
12. Name and describe its cause.
13. Describe the cultural properties of the Blastomyces farciminosus.
14. Have any other blastomyces been found in pathologic lesions in horses?
15. What is blastomycetic dermatitis in man?
16. Describe its pathologic lesions.
17. Describe the organism causing it.
18. What is Saccharomyces lithogenes and Saccharomyces granulomatogenes?

[1] A complete list of budding fungi occasionally found in pathologic lesions is given in Busse's contribution on "Sprosspilze" in Kolle and Wassermann's Manual, vol. i, p. 661.

CHAPTER XL.

BACTERIA, GENERALLY NOT PATHOGENIC, OFTEN EMPLOYED IN LABORATORY PRACTICE—BACILLI OF THE PROTEUS GROUP—BACILLUS ANTHRACOIDES—BACILLUS MEGATHERIUM—BACILLUS PRODIGIOSUS—BACILLUS VIOLACEUS—BACILLUS CYANOGENUS—MICROCOCCUS TETRAGENUS—MICROCOCCUS AGILIS—SARCINA LUTEA.

Bacilli of the Proteus Group.—Members of this group were first isolated from decaying animal material. They are aërobic and facultative anaërobic organisms which in their growth decompose proteid materials and produce a very fetid smell. They are named proteus because they are exceedingly variable in their morphology. While in their most typical shape they are bacilli of medium size, they appear, particularly in older cultures, in short coccoid form and also in curved vibrio-like shape. They stain with ordinary watery anilin solutions, but are generally Gram negative and do not form spores. The most common type of this group is the *Bacillus proteus vulgaris* of Hauser. This organism varies in length from 1.2 to 4 micra and more and is 0.6 micron wide. It is lively motile and possesses a large number of flagella distributed around the entire body. The growth on 5 per cent. gelatin is very typical. At room temperature round, depressed, whitish colonies are formed which send out wreaths of filamentous projections into the surrounding, not yet liquefied, medium. As the liquefaction goes on the peripheral portions of the growth break away from the principal mass and swarm about in the liquefied medium. This wandering away can be observed under a medium or low power of the microscope. In *firmer gelatin* of a higher concentration this swarming of broken-off portions of the colonies does not occur. The growth is best at 24° C., but it is still quite abundant at 37° C. The bacillus, when grown under anaërobic conditions, does not liquefy gelatin. Upon *agar* the bacillus forms a moist, thin, transparent growth. *Milk* is coagulated. It produces indol and phenol and reduces nitrates to nitrites. Glucose and saccharose are fermented, but not lactose. If injected in small amount subcutaneously into animals no ill effect is produced. Larger doses introduced intravenously or intraperitoneally produce death under symptoms pointing to intoxication. The Bacillus proteus vulgaris does not multiply in healthy tissues, but it can grow in necrotic tissues, and has been found in wounds. It furnishes a soluble toxin which causes

the intoxication in intravenous or intraperitoneal injection. The filtrate also contains a hemotoxin which dissolves red blood corpuscles. Meat infected with the organism has been known to cause gastro-intestinal disturbances. The bacillus if injected into the bladder of an animal causes cystitis; it has also been found in man as the cause of cystitis. The *Bacillus proteus mirabilis* is a variety of the Bacillus proteus vulgaris; it liquefies gelatin more slowly, but otherwise closely resembles the proteus. The *Bacillus proteus Zenkeri*, another variety, does not liquefy gelatin. The *Bacillus proteus Zopfii*, or *Bacterium Zopfii*, has been isolated from the intestines of chickens.

FIG. 180

Bacillus proteus vulgaris. × 1000. (Author's preparation.)

FIG. 181

Bacillus anthracoides, beginning spore formation. × 1000. (Author's preparation.)

Bacillus Anthracoides.—This organism is found in water and soil. Morphologically and in cultures it very much resembles the Bacillus anthracis, but it is no wise pathogenic. Its ends are a little more rounded than those of the bacillus of anthrax; its growth on the laboratory culture media is like that of true anthrax. Spore formation, however, is better at room than at incubator temperature. Mice and guinea-pigs can be inoculated without any evil effects. A *Bacillus pseudoanthracis* has been described by Burri. It is somewhat like the anthrax bacillus, but motile, non-pathogenic, and differing markedly in its cultures.

Bacillus Megatherium.—This organism is found on plants and in the soil and air. It is named megatherium[1] because it is a very large, plump, and very sluggishly motile bacillus. It measures 10 micra and more in length and is 2.5 micra thick, and generally occurs in short chains. Short involution forms are frequently formed in older cultures or on unfavorable media. The protoplasm of the bacillus frequently appears finely granular. It forms spores, and these leave the bacillus,

[1] Megatherium is the fossil giant sloth of South America.

not at either end, but in the equatorial plane. The bacillus megatherium is strictly aërobic; on *gelatin* the colonies are kidney-shaped or crescentic and granular after a few days' growth. The medium is liquefied in stick cultures in a funnel-shaped manner. On *agar* a whitish film is developed and on *potatoes*, a thick, smeary, grayish-white or yellowish layer.

Bacillus Prodigiosus.—This "wonderful" bacillus, so called on account of the beautiful red pigment which it forms, is a small rod, 0.5 to 1 micron long, and was formerly mistaken for a coccus. The rod shape is most marked in slightly acid media (best acidulated with tartaric acid or boric acid). The bacillus is motile, and possesses flagella, arranged on one side. It does not form spores, but can resist drying out for a considerable time; it often forms yeast-like involution forms. Pigment formation is best at 20° to 24° C. *Gelatin* is liquefied rapidly. On *agar* the colonies are at first without color, but it appears later. The pigment is only formed in the presence of oxygen. The most intense color is formed on *potatoes*. *Milk* is likewise stained red. *Sugar* is fermented by this organism.

A considerable number of different bacilli occurring in water form a red pigment, as the *Bacillus ruber aquatilis, Bacillus rubefaciens, Bacillus rubescens*, etc.

Bacillus Violaceus.—This organism has frequently been found in water. It is a motile bacillus 0.8 by 1.7 micron; it generally occurs in pairs, forms oval spores, and grows at room temperature, but not at 37° C. In *gelatin* the colonies first appear like small air-bubbles in the medium. They are irregular in outline and rapidly liquefy the culture soil. In *stick cultures* the liquefaction leads to the formation of a funnel, at the bottom of which is a violet sediment. On *potatoes* the pigment formed is of a dark, black violet; on *agar* of a bright, lacquer-like violet color. *Blood serum* is also liquefied; nitrates are reduced to nitrites. The pigment of this bacillus undergoes various changes when treated with a variety of dilute acids. Mineral acids change the violet to blue green, chlorine water to yellow, hydrate of sodium solution to brownish yellow, ammonia to bluish green. The ordinary Bacillus violaceus and a number of its varieties described are absolutely non-pathogenic. Wooley, however, encountered a Bacillus violaceus (var. Manilæ) in the Philippine Islands which appears to have caused the death of several water buffaloes, and which, after being obtained in pure cultures, was very pathogenic for rabbits.

Bacillus Cyanogenus.—This organism causes the blue discoloration of milk. It has been known for a long time, but was not obtained in pure cultures until isolated by Hüppe. It varies in size from 1 to 4 micra by 0.3 to 0.5 micron in thickness. It is motile and possesses a number of flagella at one end. It is Gram negative and grows best at room temperature, not at 37° C. It is strictly aërobic. In *milk* it does not form acid, but alkali, and does not coagulate it. The organism

forms a fluorescent pigment and a non-fluorescent-blue to blue-black pigment. The blue character of the latter is best shown in an acid medium; it is black when the reaction is neutral, brown when the reaction is alkaline. A rose-red color frequently precedes the blue pigmentation. The blue discoloration appears in milk only when it is acid, and is for this reason best produced after some development of lactic-acid bacteria. The bacillus is absolutely non-pathogenic.

Micrococcus Tetragenus.—This organism is named from the fact that it is generally found in tetrads, *i. e.*, groups of four. The individual cocci are about 1 micron in diameter. The organism is frequently found in sputum and discharges from the nose. Under these conditions it possesses a gelatinous envelope which generally surrounds the group of four. In cultures the cocci are seen singly, in pairs and as tetrads. The coccus stains easily with the watery anilin solutions and keeps Gram's stain. On *gelatin* the organism first forms small, whitish points which later develop into thick, elevated, moist drops of 1 to 2 mm. in diameter. They become confluent and finally form a continuous moist growth. Gelatin and *blood serum* are not liquefied. On *agar* the growth is not as abundant as on gelatin, but on *potatoes* it is very luxuriant. The organism is pathogenic to white mice. If small doses are injected these animals become somnolent and quiet after two days, and they die three to six days after the injection. The Micrococcus tetragenus is then found in large numbers in the internal organs of the dead mice. Gray house mice are immune; guinea-pigs develop local abscesses or a general septicemia. Intraperitoneal injection leads to a purulent peritonitis. Larger animals, such as rabbits, dogs, etc., are not susceptible.

Micrococcus Agilis.—This organism was first isolated from drinking water. It is one of the few cocci which possess a flagellum, in consequence of which they are motile. The coccus has a diameter of about 1 micron; it grows on the ordinary media at room temperature and forms a rose-red pigment. *Gelatin* becomes liquefied. The organism forms pairs, tetrads, or short chains. Its motility can best be exhibited in media containing 5 per cent. lactose. It can be demonstrated that every coccus possesses one flagellum. When several cocci adhere, particularly in tetrad form, the group generally performs a kind of rotary motion around its own axis. Loeffler and Menge have described a motile coccus forming a yellow pigment under the name of *Micrococcus agilis citreus.*

Sarcinæ.—All sarcinæ are cocci which in multiplication arrange themselves in square groups or packages which have been likened to a bale of cotton. *Sarcina lutea* is composed of comparatively large cocci about 1 micron or more in diameter. The sarcina form cannot be as readily seen in stained specimens as in the hanging drop. The growth of the organism on gelatin is at first slow and leads to the formation of point-like colonies which later become larger and

irregular in outlines and flow together forming a thick, moist, lemon-colored growth.

Sarcina aurantiaca forms an orange-yellow pigment; *Sarcina alba* a white, and *Sarcina rubra* a red pigment. *Sarcina mobilis* is remarkable for the fact that it is motile and, as claimed, possesses a flagellum. *Sarcina ventriculi* is found in the gastric contents of man and animals. The individual cocci are very large (up to 2.5 micra in diameter) and they are found in groups of eight. Whether *Sarcina ventriculi* is a definite species or not, or simply represents varieties of sarcinæ taken up with food or water, is a question not yet settled.

QUESTIONS.

1. What is the relation of members of the proteus group of bacilli to proteid material?
2. Why have these bacilli received the name proteus?
3. Describe the variability of their shape.
4. Describe the morphology and the cultural properties of Bacillus proteus vulgaris.
5. What is meant by the swarming islands of a proteus gelatin culture?
6. Has the Bacillus proteus any pathogenic properties?
7. What is the Bacillus anthracoides?
8. What is a megatherium?
9. Describe the Bacillus megatherium and state why it has received this peculiar name.
10. Describe the Bacillus prodigiosus. Why so named?
11. Are there other chromogenic bacilli which produce a red pigment?
12. Describe the Bacillus violaceus. Is it ever pathogenic?
13. Describe the Bacillus cyanogenus.
14. Describe the Micrococcus tetragenus. What does tetragenus mean?
15. Is this micrococcus pathogenic for any animal?
16. What unusual feature does the Micrococcus agilis possess?
17. What is a sarcina in general? Describe the Sarcina lutea.
18. Describe the Sarcina aurantiaca.
19. Where is Sarcina ventriculi found?

CHAPTER XLI.

INFECTIOUS DISEASES DUE TO ULTRAMICROSCOPIC VIRUSES—PLEUROPNEUMONIA IN CATTLE—CATTLE PLAGUE—HOOF-AND-MOUTH DISEASE.

It would be illogical to assume the non-existence of bacteria so small that they cannot be recognized, even with the best microscopic objectives and oculars. In fact, at least one pathogenic microorganism of this type is already known, though even under the best optical apparatuses it appears only as a small, highly refractive point. Yet from its behavior there is every reason to believe that it is indeed a bacterium. The organism referred to is the exceedingly minute microörganism discovered by Nocard and Roux as the cause of *pleuropneumonia in cattle.* In albuminous natural exudates it will not pass the Chamberland or Berkefeld filters, but it will pass these if suspended in watery solutions, such as bouillon, etc. It can be cultivated on certain artificial media and forms something like very delicate, hardly visible colonies. A further step in reasoning leads to a living virus which may or may not pass the filters in albuminous exudates, and which cannot be seen even as a highly refractive point. Another step leads to a living virus so small that it easily passes the pores of porcelain and clay filters even in natural albuminous fluids. With this the form of "contagium vivum fluidum" is reached which is designated as an *ultramicroscopic, invisible, filterable, living virus.* Some of the diseases due to such viruses which are either still on the boundary line of visibility or which are absolutely invisible and perhaps never can be seen are briefly considered in this chapter.[1]

CONTAGIOUS PLEUROPNEUMONIA IN CATTLE.

Occurrence and Historical.—Lung plague in cattle, pleuropneumonia contagiosa bovum, "Lungenseuche der Rinder" (German), peripneumonie contagieuse (French), is an acute or subacute infectious disease of cattle characterized by exudative inflammatory changes in the lungs combined with sero-fibrinous pleuritis. The disease

[1] Helmholtz, the celebrated German physicist, has shown that in consequence of the inherent properties of light, it will be impossible, no matter how much the microscope is improved, to see objects smaller than a certain minimum which he calculated exactly. There is of course, no reason whatever for assuming that there are no live objects in nature beyond this microscopic limit.

is caused by the smallest known bacterium, which was discovered by Nocard and Roux in 1898. The epizoötic was first correctly described by Bourgelat in France (1765), but it had previously been noticed in Germany. It has prevailed throughout Europe and was imported to Africa, Asia, and Australia, and introduced into the United States, probably first into New York in 1843. It then spread to various States, but it was vigorously opposed and finally stamped out in 1891, since which time this country has been free from the disease.

Pathologic Lesions.—The principal lesions are generally found only on one side of the thorax and on the pleura of the same side, but there appears to be no predilection as to the side involved. This is true of 75 per cent. of the animals affected. According to Nocard, the pleura is always involved, but in very variable degrees. Sometimes when the inflammatory changes in the lung greatly predominate a certain amount of thickening and infiltration is seen in the pleura pulmonalis of the same side. In a more advanced stage there is vascularization of the serous membrane, with an abundant fibrinous exudate on its surface. The lungs in acute cases are found hepatized in more or less extensive areas; they are void of air and non-elastic. On section a clear, serous, yellowish fluid oozes out of the areas of hepatization. When collected in a clean vessel this fluid subsequently coagulates into a gelatinous mass. The interlobular connective tissue is increased and forms a light yellowish network which divides the hepatized area into irregular patches of various colors. The latter may be gray, light red or dark brown red, so that a cut surface presents a mottled appearance. The increased interalveolar connective tissue shows enlarged lymph vessels and clefts filled with a yellowish serous or a more fibrinous exudate. The obliterated alveoli of an area of hepatization are sometimes light red and firm at the periphery, while the centre of the solid tissue has become dark red and soft-elastic. The walls of the bronchi in the affected pulmonary areas are infiltrated, their lumina contain an exudate and the peribronchial and mediastinal glands are quite edematous. While the pleura over the diseased portions of lungs generally shows the changes described above, it may also contain very little fluid between the visceral and the parietal layer or, on the other hand, a large amount of clear yellow fluid or a yellowish-gray cloudy fluid with flocculi of fibrin may be present. When only very small amounts of pleuritic exudate are present the condition is known as *pleuritis sicca.* On the other hand the amount of fluid may be 15 to 20 liters. In some cases the greater portion of the exudate is not found free between the two layers of the pleura, but has infiltrated the mediastinal connective tissue. The latter then forms a soft gelatinous tumor composed of confluent, infiltrated masses of a yellow color. If cut into, these masses discharge an abundant amount of yellowish or decidedly amber-colored serous fluid. The description

as far as given refers more particularly to the acute form of the disease.

In the more chronic form there is considerable fibrosis of the affected lung, *i. e.*, new formation of inflammatory connective tissue. Areas in the lung appearing almost as solid as meat (a condition known as *carnification*) alternate with necrotic and occasionally *calcified areas.* The necrotic portions, or sequesters, may assume a considerable size and may bulge as nodular masses above the general pleural surface. The sequesters are frequently surrounded by a tough, fibrous, connective tissue capsule and discharge, after an incision through the latter, a mushy, dirty mass and more solid particles. Sometimes such softened, necrotic portions of the lung break into a bronchus and are discharged through it into the outside world. They vary in size from a walnut to a child's head, and they may be formed after a preliminary purulent infiltration, or the serous yellowish exudate may so compress bloodvessels and lymphatics that portions of the lung are deprived of their nutrition and necrosis results from the lack of blood supply.

Histologic studies by Pourcelot and MacFadyean have shown that the first tissue changes occur in the interalveolar septa and that their lymphatics become distended with a finely granular fibrinous coagulum containing a few cells. The alveoli at this early stage show no marked changes, a little later, however, the zone adjacent to the alveolar wall secretes a fibrinous exudate which extends toward the centre. At the same time the interalveolar connective tissue becomes infiltrated with leukocytes and subsequently thickened by newly formed connective tissue. The mucosa becomes covered with a fibrinous exudate. The changes in the pleura first consist in an edematous infiltration, later the surface becomes covered with a fibrinous exudate, false membranes are formed, and these finally become organized and replaced by cicatricial connective tissue. The pericardium is sometimes involved and the peritoneal cavity occasionally contains some fluid and an exudate upon its surface.

Bacteriology.—Nocard describes the bacteriology of the disease as follows: The examination of stained specimens from the virulent serous exudate infiltrating the alveoli does not show any bacteria. This serous fluid, however, when properly preserved or mixed with bouillon, does not lose its virulency. In spite of the fact that it apparently contains no microörganisms, its inoculation into a place as unfavorable as the end of the tail of a cow produces considerable reaction and sometimes death. If heated to from 65° to 70° C. the exudate loses its virulency. This also occurs after certain processes of filtration. If *collodion sacs,* inoculated with a trace of the exudate obtained under aseptic precautions, are implanted into the peritoneal cavities of rabbits and re-obtained after fifteen to twenty days, the contents of the sacs have become opalescent and slightly cloudy. Upon microscopic examination of the contents neither cells nor bacteria

are seen which could be cultivated upon the ordinary culture media. Examination, however, with very good objectives and strong light at a magnification of 1500 to 2000 diameters, show innumerable very small motile and highly refractive *points*. These are live organisms which, protected against phagocytosis by the collodion sac,[1] have been able to multiply.

They can also be cultivated outside of the living body, in a special *bouillon* prepared according to Martin, as follows: Peptone from digested pig's stomach, 5 parts to 100 parts of cattle or rabbit serum. This must be freed from bacteria by filtration through a Pasteur filter, but must not be heated. If this medium is inoculated from collodion sacs and incubated at 37° C. for two or three days it becomes opalescent, and if shaken there is evidence of slight clouding. The highest magnifications show exceedingly minute highly refractive granules, so small that no conclusions as to their shape can be drawn. These cultures are, however, virulent and they can also be used for immunizing inoculations. Solid culture media can be prepared by adding sterile melted gelatin to Martin's liquid medium. If a drop of pulmonary exudate or of fluid culture is allowed to run over the surface of the gelatin a large number of very delicate transparent colonies can be seen after three or four days with the aid of a microscope. A little later the colonies become denser and opaque in the centre. They finally cover the surface with a veil which adheres so firmly that it can only be removed by breaking up the culture medium. If the albuminous exudate or a culture in Martin's liquid medium is filtered through a Chamberland or Berkefeld filter a sterile fluid is obtained; if, however, the fluids are diluted with a watery liquid the microbes pass through. The latter fluid is fully virulent. The organism is *aërobic;* it does not multiply *in vacuo* or in inert gases; the growth is always slow and scanty, and its optimum temperature is at 36° to 38° C. The microbe takes the basic aniline stains, but it is so small that even after prolonged staining with strong solutions the shape cannot be ascertained and the fine colored dusty particles which are seen are apparently each composed of numerous individual microbes. If *impression preparations* are made the mass on the cover-glass takes the stain. It is decolorized if treated by Gram's method.

Animals Susceptible.—Cattle, buffaloes, yak, and bison are the only susceptible animals. Natural infection occurs by close contact between cattle. While there is some evidence that infection occurs

[1] The fluid to be inoculated into collodion sacs is obtained in the following manner (Nocard): It is necessary to procure a lung from an animal in an early stage of the acute form of the disease. A place is selected which indicates a consolidation and a collection of fluid behind the pleura. The latter is then superficially cauterized with a hot knife or steel spatula. A fine pointed, sterile, glass pipette is pushed through the cauterized pleura into the layers of the tissue next to it, and about 0.5 c.c. of the clear amber-colored fluid may be so obtained. From this collodion sacs containing sterile bouillon are inoculated, and these after having been sealed at the glass end are implanted into the peritoneal cavity of rabbits. Martin's medium may also be inoculated from the amber fluid obtained by puncture of the pleura.

by inhalation, experiments to spread the disease by this method have generally been unsuccessful. The virus may remain alive and active for some time in stables where sick animals have been kept and it may spread to new importations even after the disease has disappeared among the stock by deaths or recoveries. If fluid containing the microörganism of the disease is inoculated in a very small dose into the subcutaneous connective tissue of cattle an edematous, painful swelling appears after a variable number of days at the point of inoculation, the temperature rises to 41° to 42° C., and death occurs after a fall of temperature and a comatose condition have set in.

Protective Inoculation.—This has been practised for many years, first in an empirical manner, and in 1852 by Willems, who dipped a vaccination lancet into the juice expressed from the alveoli of an infected lung, and vaccinated in two or three places at the lower end of the tail. Pasteur, in 1881 and 1882, used a lymph attenuated by mixing the fluid from affected lungs with glycerin. A large number of investigators have at various times attempted to devise an effective protective inoculation. Raebiger, however, who has critically reviewed the various methods, comes to the conclusion that their protective value is problematical, that the danger of contraction of the disease by non-immune animals from vaccinated animals is great, and that the best method of limiting the disease consists in the slaughter of all infected animals. If for any reason this is impossible, protective vaccinations by attenuated lymph or artificial pure cultures may be practised.

CATTLE PLAGUE.

Occurrence.—Cattle plague, contagious typhus, pestis bovina, or Rinderpest (German), is a highly contagious, very fatal (60 to 90 per cent. mortality) disease of cattle. It is very prevalent in various parts of Asia, including the Philippine Islands, Africa, and is also met with in Europe, in the southern parts of the Balkan peninsula, and in southern Russia. The organism causing the disease is not known, but it is probably of a type similar to that of contagious pleuropneumonia. It appears that the virus contained in natural albuminous body fluids does not pass the Chamberland or Berkefeld filters, but that it does when such albuminous fluids are diluted with a sterile watery fluid.

Pathologic Lesions.—The most characteristic pathologic lesions of the disease which has a rapid onset are strong hyperemia of the conjunctiva, fetid discharge from the nostrils, and hemorrhagic diarrhea. On postmortem examination the fourth stomach is found strongly hyperemic, with petechiæ on the elevated margins of the folds of the mucosa, and in the more chronic cases ulcerations are formed. The intestines are covered with a fibrinous exudate, which

sometimes forms a complete cast of the gut. Peyer's patches and the solitary follicles are swollen and hyperemic. Sometimes the serous membranes, particularly the pericardium and the pleura, likewise show hemorrhages. The liver and spleen are not markedly enlarged, though much congested. In certain localities epizoötics have occasionally also shown hemorrhages and ulcerations in the buccal and nasal mucosa.

Immunization.—Since it had been noticed that animals which had recovered from the disease were immune, attempts at immunization were early undertaken. The first experiments of this kind were made by Semmer and Nencki. Koch, Kolle and Turner, and Theiler and others have worked out methods of preparing a "Rinderpest" immune serum of high value. This is done in the following manner: Healthy cattle which must particularly be free from piroplasmosis and trypanosomiasis are first immunized by the simultaneous method. On one side of the body they receive 1 c.c. of virulent blood from an animal sick with cattle plague and on the other side a dose of 15 to 55 c.c. of an immune serum. An immune serum of high value is subsequently furnished only by those animals which react to this simultaneous injection by fever and otherwise. Races of cattle which show no reaction are unsuitable for the preparation of an immune serum. After an animal which has reacted has recovered it receives an injection of 100 c.c. of virulent blood. This is again followed by fever. When the animal has fully recovered it receives 200 c.c. of virulent blood. This method of giving gradually increasing doses is continued until 1000 c.c. of virulent blood have been injected at one time. After the last reaction has subsided the animal is bled on three successive days or on three days with an interval of one day between. Each time 4500 c.c. are withdrawn. After a short interval the animal can again be injected with a large dose of virulent blood and also be bled again. In this manner it is possible to obtain large amounts of the immune blood. The latter is allowed to coagulate, the serum is collected and a sufficient quantity of 5 per cent. carbolic acid is added to it so that the mixture contains 0.5 per cent. of carbolic acid. In the preparation of the immune serum it is necessary always to have on hand fresh virulent blood. This is obtained by injecting 0.1 to 0.05 c.c. of very virulent blood into unprotected but perfectly healthy cattle. Koch has shown that sheep are also susceptible to cattle plague if inoculated with virulent blood, though they rarely die from the disease, and for this reason they may be used to furnish the virulent blood. The titre, or immunizing value of the immune serum, is determined by injecting non-immunes with 1 c.c. of virulent blood and estimating what quantity of the immune serum will protect against this dose.

Kolle and Turner have ascertained that a good immune serum will protect cattle for three months against "Rinderpest" in a dose of 100 to 200 c.c., and that, particularly in still larger doses, it will save cattle

already in the early stage of the disease. Immune serum, if properly preserved, retains its protective and curative properties for several years; in one observation for five years.

AFRICAN HORSE SICKNESS.

This affection, also known as pestis equorum, "Pferdepest" (German), peste du cheval (French), is an acute or subacute disease of horses, prevalent in epizoötic form in Africa. As first demonstrated by McFadyean and afterward by Nocard, it is due to an ultramicroscopic, filterable virus. Theiler, Eddington, and Koch have studied the disease, with the object of preparing a protective serum. The most important pathologic changes of the disease are gelatinous infiltrations of the subcutaneous and intermuscular connective tissue, swelling of the superficial lymph glands, very marked catarrhal swelling of the mucosa of the stomach, and the first portion of the small intestine, edema of the lungs, and occasionally the formation of gelatinous deposits on the pleura, pericardium, and epicardium. One attack of the disease produces a relative immunity only, and animals which have recovered from the disease may again be made very sick by the injection of large doses of virulent blood. Koch used horses which had passed through a natural infection (so-called "salted" horses) in the preparation of an immune serum of high value. It is obtained by injecting into "salted" horses increasing doses of virulent blood obtained from sick horses shortly before death. According to Theiler the protective inoculation consists in the injection of 300 c.c. immune serum into the jugular vein and of a small amount of virulent blood under the skin.

FOOT-AND-MOUTH DISEASE.

Foot-and-mouth disease, also known as aphthæ epizoöticæ, aphthous fever, infectious aphtha, eczema epizoötica, "Maul und Klauenseuche" (German), "fièvre aphtheuse" (French), is an acute highly contagious fever, due to a specific contagium. It is characterized by the eruption of vesicles or blisters in the buccal mucosa, around the coronets of the feet, and between the toes. It is most commonly a disease of cattle, but it also affects swine, sheep, goats, buffalo, bison, deer, etc., and less frequently horses, dogs, and cats. Man may be infected by contact with sick animals or by drinking their raw milk.

The contagium, an ultramicroscopic filterable virus, is contained in the contents of the vesicles, in the saliva and milk, and during the height of the disease, also in the blood serum. While the mortality from the disease is generally not very high, epidemics are so wide-

spread and lead to so considerable a loss in milk that the total loss is relatively large. The disease is very prevalent in Europe and has, on several occasions, invaded the United States. It may enter a free territory in a very insidious manner and spread before it has been recognized. The most recent outbreak in this country, discovered in October, 1908, by Pearson, in Pennsylvania, and described in Circular 147 of the Bureau of Animal Industry, by Mohler and Rosenau, furnishes an interesting instance of the mode of spreading. The disease was found to have been introduced into the United States with a smallpox vaccine imported from Japan. The vaccine had been used and propagated by one manufacturing firm for a considerable time and no outbreak of hoof-and-mouth disease occurred, because the inoculated calves were slaughtered immediately after the vaccine had been obtained. A second firm, however, afterward obtained vaccine from the first, used it in the inoculation of calves, and sold these animals after the crop of lymph had been collected. It was through these calves that the hoof-and-mouth disease had been spread to a number of places before it was discovered. Fortunately the Government authorities traced all the foci of infection and stamped out the epizoötic before it had reached any great proportions. The conclusions drawn by Mohler and Rosenau and the others who took part in the investigation are the following:

1. The recent outbreak of foot-and-mouth disease in this country started from some calves used to propagate vaccine virus.

2. The vaccine virus used on these calves has been proved to contain the infection of foot-and-mouth disease.

3. The outbreaks of foot-and-mouth disease in 1902–3 probably had a similar origin.

4. It is probable that the foot-and-mouth infection got into the vaccine virus in some foreign country in which the disease prevailed, and was introduced into the United States through the importation of this contaminated vaccine.

5. The symbiosis between the infections of vaccinia and foot-and-mouth disease is especially interesting. Animals vaccinated with the mixed virus, as a rule, show only the lesions of one of these diseases, namely, vaccinia; nevertheless the infectious principle of foot-and-mouth disease remains in the vaccinal eruption.

Protective Inoculation.—This has been practised empirically for a long time. Since the disease spreads through a large herd rather slowly, and may thus cause a prolonged quarantine, stock owners have been in the habit of inoculating their cattle after an outbreak by rubbing saliva from infected animals into the mucous membrane of healthy ones, or contaminating rough fodder with the infected saliva. This method, however, is only applicable when the epizoötic occurs in a milder form. The severe form of the disease, when propagated in this manner, is likely to cause considerable loss. Loeffler, Frosch, Uhlenhuth and Hecker, and others, have, for a number of years, been

engaged in attempts to devise an effective method of protecting cattle against hoof-and-mouth disease by injecting simultaneously an immune serum and virulent lymph, but up to the present time no efficient method seems to have been worked out. The last method recommended by Loeffler requires four different inoculations. Casper, who has reviewed the subject, concludes that there is yet no method of appreciable value in practice, and that the whole subject is still in an experimental stage.

DISTEMPER IN DOGS.

Distemper in dogs, dog plague, dog ill, "Hundeseuche" (German), Pasteurellosis canum, "Maladie du jeune age" (French), is an acute, contagious disease of young carnivora, particularly of dogs, characterized by an acute catarrh of the upper respiratory passages, often associated with a catarrhal pneumonia. In fatal cases the mucous membranes of the nose, larynx and bronchi are reddened, swollen and covered with an abundant fibrinous exudate. The finest bronchioles are filled with a purulent material and the pulmonary parenchyma exhibits smaller or larger areas of consolidation. The pleura of such diseased portions of lung is often covered with a slight fibrinous exudate. The gastric and intestinal mucosa is swollen and reddened, the mediastinal and mesenteric glands are swollen. The heart, liver, and kidneys show evidences of parenchymatous degeneration. In some cases the spinal cord shows disseminated inflammatory foci (myelitis disseminata). Lignière claimed to have discovered a bipolar bacillus (Pasteurella canis) as the cause of dog distemper, but his claim has not been confirmed. Jess also claimed the discovery of a bacillus as the cause of distemper, but his work likewise has not been confirmed. It appears rather that the disease is due to an ultramicroscopic filterable virus. None of the vaccines and sera against dog distemper which have from time to time appeared have proved efficient.

FOWL PLAGUE.

Fowl plague, pestis gallinarum, pestis avium, "Hühnerpest" (German), "peste aviaire" (French), is an acute contagious disease of chickens, which has formerly generally been confounded with chicken or fowl cholera. It is, however, a distinct disease, not due, like chicken cholera, to a bacterium of the hemorrhagic septicemia group, but to an ultramicroscopic filterable virus. The latter is present in the blood, the secretion from the nose, the feces, the serous exudates, and the bile of sick birds. Infection can be produced artificially by inoculation of exceedingly small doses of blood (0.000001 c.c.). Blood kept in fused glass tubes in a cool, dark

place can retain its virulency for three months. In very acute cases of this disease which has been observed in several European countries the only pathologic changes found are hemorrhages into the serous membranes; in less acute cases there is a marked edema of the subcutaneous connective tissues and a collection of pale yellow fluid into the serous membranes and a fibrinous exudate upon them. The disease, therefore, has also been called typhus exudativus gallinarum.

CHICKEN-POX.

Chicken-pox, epithelioma contagiosum avium, "Geflügelpocke" (German), "la petite verole" (French), is a chronic contagious disease of fowl, characterized by the formation of epithelial nodules on the comb and neck and on the mucous membranes of the head. It is caused by a filterable virus. The latter can be obtained by grinding up the epithelial proliferations, mixing the mass with some sterile physiologic salt solution, and filtering the mixture through porcelain or clay filters. The disease, after natural infection, begins with the formation of grayish-red, shiny patches in the places indicated. The small, slightly elevated patches soon increase in size, become more elevated and grayish yellow in color. They finally form nodules of the size of a pea or larger, dry and warty in character, and containing in their interior a yellowish fatty mass. The wart-like masses sometimes become confluent and cover entirely larger areas of skin or mucosa. The disease generally ends in recovery by drying up and shedding of the epithelial proliferations. If, on the other hand, the affected animals die it is found that the process has extended from the mouth toward the larynx and in it and the bronchi a mucopurulent exudate and caseous lumps are found. The lungs contain bronchopneumonic inflammatory foci. The intestinal mucosa is inflamed and studded with small hemorrhages. The histologic changes of the skin and mucous membranes of the mouth consist in a hypertrophy of the epithelial covering, a proliferation of the interpapillary pegs with congestion of vessels and inflammatory cell infiltration in the derma.

Bollinger and Coskor claim that epithelioma contagiosum of man and of fowl is identical and that it can be experimentally transferred from the former to the latter. The disease described and commonly known as chicken-pox in fowls has, of course, nothing in common with the disease known as chicken-pox in man, the latter being one of the febrile exanthematous contagious diseases, characterized by a vesicular eruption over the entire body.

QUESTIONS.

1. What is contagious pleuropneumonia in cattle? Where does the disease occur? Has it ever been seen in the United States?
2. Describe its pathologic lesions in the lungs.
3. Describe its changes produced in the pleura.
4. What is meant by a carneous lung?
5. Which material contains the infectious virus?
6. Describe the formation of necrotic pulmonary sequesters in the disease.
7. Describe the histo-pathologic changes in the lungs and pleura.
8. What is a pleuritis sicca?
9. Describe the method of obtaining clear, uncontaminated virulent serous fluid.
10. What does microscopic examination of this fluid show?
11. How can the organism of pleuropneumonia be cultivated?
12. How do its cultures look in collodion sacs? How on Martin's gelatin-serum medium?
13. How is the latter prepared?
14. Under what conditions does the virus of pleuropneumonia pass a Chamberland filter? under what conditions does it not pass?
15. Discuss protective inoculation against pleuropneumonia.
16. What is Rinderpest?
17. What kind of an organism causes it?
18. Describe the most characteristic pathologic lesions of the disease.
19. Describe the Kolle-Turner method of preparing a rinderpest immune serum of high value.
20. What method is used to have on hand large amounts of virulent blood?
21. What are the curative properties of a high-value immune serum? What are its properties as to stability?
22. What is African horse-sickness due to? What are its most characteristic pathologic lesions?
23. What is hoof-and-mouth disease in cattle?
24. How may the disease be spread by cowpox vaccine lymph?
25. What methods of protective inoculation have been practised? Discuss their value.
26. What is the cause of distemper in dogs? What are the most characteristic anatomic lesions?
27. What is the cause, what are the pathologic lesions of fowl plague?
28. Describe the pathologic lesions of chicken-pox, or epithelioma contagiosum avium?

PART III.

MICROÖRGANISMS IN FOODS AND SOILS.

CHAPTER XLII.

THE BACTERIA OF MEAT-POISONING—BACILLUS ENTERITIDIS—BACILLUS BOTULINUS.

MEAT from healthy animals, obtained in a clean manner, is free from bacteria. At first they are found on the external surfaces only, and after a variable period of time, depending upon temperature and other environmental conditions, they may penetrate to a certain distance into the interior of the meat. If kept for a longer period and at a higher temperature, putrefactive saprophytic bacteria develop throughout and cause it to become putrid. Such meat may often be ingested by animals and occasionally by man without necessarily producing disease. On the other hand, meat from animals which have been slaughtered on account of disease may appear in every way normal, and yet may produce violent disease and even death when eaten. In some cases only raw or very insufficiently cooked or boiled meat produces disease, but in other cases disease also follows the ingestion of meat which has been well cooked. The study of meat-poisoning epidemics in various parts of the world has shown that sickness generally follows the ingestion of meat from animals suffering from severe gastro-intestinal disturbances or general septic conditions. In a number of examinations made on meat of this kind the blood-vessels were found full of bacteria, which when obtained in pure cultures proved highly pathogenic to animals. Bollinger was probably the first observer who held that such intoxication after the use of meat was due to the intestinal septic conditions from which the animal that furnished the meat had suffered itself.

BACILLUS ENTERITIDIS OF GÄRTNER.

Morphology and Historical.—Gärtner was the first to isolate a bacterium as the cause of a meat-poisoning epidemic following the use of meat from an emergency slaughtered sick cow. Since then

Van Ermengem, Gaffky and Paak, Neelsen and Johne, and others, have also studied similar outbreaks. They found a bacillus acting through a poison which is not even destroyed by boiling the infected meat, as the cause of a certain form of meat-poisoning. This organism is known as the Bacillus enteritidis of Gärtner. It belongs to the typhoid-colon group. It is a short bacterium, and is frequently of ovoid form, not longer than 0.2 to 0.4 micron, and generally arranged in pairs. It often stains unequally, more at the poles, and then resembles an organism of the hemorrhagic septicemia group. It is Gram negative, motile, and provided with four to eight, sometimes ten or twelve long flagella. These cannot be well stained by Loeffler's method, but they can be demonstrated by the silvering method of Van Ermengem.

Cultural Properties.—The superficial colonies on *gelatin* are variable in form and often cannot be distinguished from the colonies of the Bacillus coli, though they are generally more transparent and more regular at the periphery. The organism does not form indol or in traces only. It does not coagulate *milk*, but causes it to become decidedly alkaline after ten days, and also more transparent and yellowish in color. It ferments glucose, lactose, galactose, maltose, saccharose, and also glycerin. *Bouillon* becomes rapidly clouded, and an easily torn pellicle is formed on the surface. It does not produce a fecal smell like the colon bacillus. The growth on *potatoes* varies. It is sometimes invisible like that of the typhoid bacillus, at other times dirty yellowish or brownish like that of the colon bacillus. It grows quite well in Petruschky's *litmus whey* without much acid production or color changes. In Rotberger's *neutral-red agar*, containing 0.3 per cent. glucose, the color disappears and gas is produced after eighteen to twenty-four hours. On the *Drigalski-Conradi medium* bluish colonies somewhat larger and less transparent than those of the tyhoid bacillus are formed after sixteen to eighteen hours.

Toxin Production and Other Properties.—The Bacillus enteritidis is distinguished from all other bacteria of the typhoid-colon group by its property of forming diffusible toxins, which are very resistant to higher temperatures. The bacilli, when introduced in small quantities into the stomachs of small animals or injected subcutaneously intravenously or intraperitoneally have a fatal effect. Such susceptible animals are mice, guinea-pigs, rabbits, monkeys, calves, etc. The organism produces local inflammatory conditions in the serous membranes, and also a general septicemia, and it can be found in considerable numbers, particularly in the muscles. The toxin, even if it has been heated to the boiling point of water, produces the same pathologic lesions in the intestines, the kidneys, and in the serous membrane in general. Whether the Bacillus enteritidis is a single species or whether there are several nearly related varieties is a point not yet fully settled. It appears that at least one of the varieties of

meat-poisoning bacilli described belongs to the group of paratyphoid bacilli. These are several varieties of bacilli which closely resemble the Bacillus typhosus.

Agglutination.—It has been shown that the blood serum of patients suffering from meat poisoning has a comparatively high agglutinating power of 1 to 75–400 toward the Bacillus enteritidis, isolated from the meat which had caused the sickness, while the serum from healthy persons agglutinates in a proportion of 1 to 25 only. It has, on the other hand, also been demonstrated that the serum from typhoid hyperimmunized animals also agglutinated the Bacillus enteritidis in very high dilutions, but it is never agglutinated in as high dilutions as the typhoid bacillus itself. De Noble observed that a typhoid immune serum which agglutinated the typhoid bacillus in 1 to 30,000 would agglutinate the Bacillus enteritidis in 1 to 2000. Fischer showed that an immune serum of the Bacillus enteritidis which would agglutinate it in 1 to 40,000 would agglutinate the Bacillus coli only in 1 to 10. This shows that the Bacillus enteritidis is certainly not a variety of the colon bacillus. Various stems of hog-cholera bacilli behave toward such an immune serum like very distinct varieties and not like one identical species.

Infection.—Infection with the Bacillus enteritidis may occur through other sources than meat from sick animals containing this organism. Probably it may also be conveyed by milk and by contact between sick and healthy persons. It appears also that an organism described by Petruschky under the name of Bacillus fecalis alkaligenes obtained from the feces of a patient sick with a typhoid-like disease is identical with the Bacillus enteritidis.

Prophylactic Measures.—To protect the public against the use of meat which might cause infection with the Bacillus enteritidis the following measures are recommended: Small pieces of meat are removed with sterile instruments 2 or 3 cm. below the external surface. With these pieces agar tubes are inoculated. They should remain sterile, since the interior of meat from healthy animals does not contain any bacteria. Another method was proposed by De Noble. A piece of meat is obtained from the interior and its juice is expressed and diluted to the proportion of 1 to 10 to 20 with physiologic salt solution. With this fluid agglutination tests are made with emulsions of cultures of the Bacillus enteritidis. If the juice agglutinates in a dilution of 1 to 10 to 20 it shows enteritidis infection of the meat, because the juice from healthy meat does not agglutinate in a proportion of 1 to 1. If cultures are to be obtained from infected meat it is well to keep it for twenty-four hours at 18° to 20° C. Under these conditions the enteritidis bacillus multiplies rapidly in the interior of the infected meat.

Meat poisoning has also been observed from the use of meat already somewhat putrid and containing large numbers of colon or proteus bacilli.

BACILLUS BOTULINUS.

There is still another form of meat poisoning due to a bacterium, the toxins of which are destroyed by heating. This form of meat poisoning is known as *botulismus*, or *allantiasis*. It has been most frequently observed following the ingestion of sausages and pickled or smoked meat. Similar affections have been traced to the consumption of fish and they are then spoken of as *ichthyosismus*. Articles of food through which these intoxications are produced, are generally prepared and kept under conditions which favor the development of anaërobic bacteria; they are consumed raw or imperfectly boiled or cooked. The symptoms of botulism differ from the gastro-intestinal disturbances characteristic of Bacillus enteritidis infection or intoxication and point to nervous disturbances, such as paralysis of the muscles of the eye, double vision, constipation, and enuresis. Diarrhea and vomiting are rare, and if present they are of a transitory character. Other disturbances often noticed are dryness of the buccal and pharyngeal mucosa and redness, disturbance of the voice, and difficulty in respiration.

Morphology.—The *Bacillus botulinus* was discovered in 1895 in a meat-poisoning epidemic affecting 50 persons by Van Ermengem, who investigated it most thoroughly and furnished the following description: It is a strictly anaërobic bacillus, 4 to 6 micra long and 0.9 to 1.2 wide; its ends are somewhat rounded off, it forms groups of two or somewhat longer chains; it is sluggishly motile and possesses four to eight very fine flagella, arranged in a peritrichous manner.

Cultural and Staining Properties.—It stains easily and is Gram positive. The growth is most characteristic on *glucose gelatin plates*, where the young colonies are circular, transparent, slightly yellowish, and composed of coarse granules, which show a constant motility. Zones of liquefied gelatin surround the colonies. The colonies later become larger, brown in color, and opaque, and only the granules at the periphery retain their motility and look like thorny projections. The latter become larger and divide and form digit-like masses and the entire colony resembles a sunflower. Stick or stab cultures in *glucose gelatin* or *agar* are not as characteristic. These media become cleft and broken up in consequence of abundant gas formation. The liquefied gelatin later becomes transparent because the growth falls to the bottom as a whitish, flocculent sediment. A large amount of gas composed of hydrogen and methane (CH_4) is formed. All cultures give off a smell of butyric acid, but there is no marked fetor. *Milk* is not coagulated; lactose and saccharose are not fermented. The growth is abundant under anaërobic conditions between 18° to 25° C.; at 37° to 38.5° C. the growth is scanty, involution forms soon appear, and toxins are no longer formed. In *bouillon cultures* kept at 37° to 38.5° C. the organism forms long, intertwined filaments. The bacillus at medium temperatures forms oval, somewhat elongated, endogenous

spores. It requires an alkaline medium, no growths occur on acid media, and 5 to 6 per cent. of sodium chloride also prevents its multiplication. The spores are not very resistant and are destroyed if exposed one hour to 80° C. They are killed by 5 per cent. carbolic acid within twenty-four hours. If protected against air and sunlight they remain alive for a long time either in moist or desiccated condition. The organism, however, dies comparatively quickly in *glucose gelatin* and the cultures must be transferred every three or four weeks. It is also necessary to transplant a larger mass of the sediment from such cultures. The culture media should always be distinctly alkaline and not exposed to temperatures above 25° C.

Animals Susceptible.—If small amounts of cultures (0.0003 to 0.001) are injected into rabbits the animals may die within thirty-six to forty-eight hours. Sometimes they live three to four days, then become paralytic and die shortly after these symptoms have appeared. If the cultures are introduced into the stomach the effect is slow and uncertain. Doses of 5 to 10 c.c. of a bouillon culture may kill rabbits within forty-eight hours. At times a slow cachexia develops, and the animals only die after a number of weeks. Guinea-pigs are very susceptible to minimal doses; they show aphonia, forced respiration, great dyspnea, and death. Mice and monkeys are not very susceptible. Dogs, chickens, and cats are refractory, and can withstand large doses given repeatedly, but they may show some transitory paretic symptoms.

Toxin Production.—Van Ermengem believes that the symptoms due to the Bacillus botulinus in man and in artificial animal inoculation are due exclusively to intoxication without any multiplication of the bacillus in the infected organism. He classifies the Bacillus botulinus as a *pathogenic* or *toxigenic saprophyte,* and likens its action to that of the large poisonous fungi which produce disease and death by the amount of already formed toxin introduced with them into a susceptible animal. The *botulismus toxin* which affects susceptible animals not only in subcutaneous or intravenous injections, but also if introduced into the stomach, is not very resistant. It is destroyed if heated to 80° C. for one hour; it is almost instantaneously destroyed by a 3 per cent. carbonate of soda solution; it is somewhat more resistant toward acid and soon succumbs to the effect of light and air. This toxin, like the tetanus toxin, is fixed by emulsions from the central nervous system, by lecithin, cholesterin, fatty substances. Van Ermengem recommends the following as prophylactic measures against infection with the Bacillus botulinus: Articles of food which in a raw condition may be easily decomposed, such as ham, sausages, salted fish, should not be eaten raw. All foods which give off a smell of butyric acid are suspicious and may contain the Bacillus botulinus. Pickled meat or fish should be soaked before use in a not less than 10 per cent. salt solution, which will destroy the Bacillus botulinus.

Kempner has prepared an antitoxic botulinus serum which proved efficacious in protecting animals.

QUESTIONS.

1. Discuss the bacterial contents in meat from healthy animals.
2. Under what circumstances are bacteria found in the vessels in the interior of the meat of food animals?
3. May such bacteria containing meat be dangerous after cooking?
4. Who discovered the first meat poison organism?
5. Describe its morphology.
6. Describe its cultural characteristics.
7. Differentiate between the cultural features of Bacillus enteritidis and Bacillus coli.
8. What symptoms does the Bacillus enteritidis cause in man if ingested with infected meat?
9. What is the behavior of the blood serum of persons suffering from Bacillus enteritidis infection toward this organism?
10. How does a typhoid-immune serum of very high value behave toward the Bacillus typhosus and the Bacillus enteritidis?
11. What is the Bacillus fecalis alkaligenes?
12. What methods have been recommended to detect the infection of meat with the Bacillus enteritidis?
13. What is botulismus, or allantiasis?
14. What is ichthyosismus?
15. Can botulismus be contracted from well-cooked articles of food? If not, why not?
16. What are the symptoms of botulismus in man?
17. Describe the morphologic properties of the Bacillus botulinus.
18. Describe its cultural characteristics on glucose gelatin plates.
19. Describe the spores and discuss their resistance.
20. What is the optimum temperature of the Bacillus botulinus?
21. Describe its pathogenic properties toward laboratory animals.
22. What is meant by a pathogenic or toxigenic saprophyte?
23. Discuss the resistance of the botulinus toxin.
24. What are its features of similarity with the tetanus toxin?
25. What measures does Van Ermengem recommend to prevent botulismus?

CHAPTER XLIII.

BACTERIA OF THE NITROGEN CYCLE—FERMENTATION OF UREA —NITRIFYING AND DENTRIFYING ORGANISMS— FREE NITROGEN FIXATION.

THE importance of the nitrogen cycle in its relation to the maintenance of vegetable and animal life has already been briefly referred to in Chapter II. It was there stated that bacteria and other low vegetable organisms perform a most important function of this cycle. The subject will now be taken up in greater detail in the discussion of the particular bacteria concerned in these phenomena.

Plants, which are ultimately necessary for the preservation of animal life, obtain their nitrogen from the soil, chiefly in the form of nitrates (*i. e.*, salts of nitric acid), and to only a small extent in the form of ammonia salts. As the amount of nitrates in the soil is limited, it would soon become sterile if the nitrates were not replaced. The nitrates taken up by the higher plants are changed in their metabolism into vegetable proteids, such as the gluten of grasses and the legumen of leguminosæ (clover, peas, beans, lentils, etc.), and these in turn when taken up as vegetable food are changed into other proteids in the animal body. Man and the lower animals excrete the nitrogen waste mainly in the form of urea. Lafar states that mankind excretes daily about 37,500 tons of urea, containing seventeen million kilograms of combined nitrogen, while animals certainly excrete a much larger daily amount of this nitrogenous waste product. Urea cannot be directly utilized by our cultivated plants and the question, therefore, arises, How does this animal nitrogen-containing waste product become available again as a source of nitrogen for the building up of vegetable proteids? It is through the decomposition of the urea by the action of bacteria and the change of this decomposition product by other bacteria, making it, finally, again utilizable in the nutrition and metabolism of plants. By decomposition is meant the breaking down of bodies of a more or less complex chemical composition into simpler compounds; in other words, the changing of a complex molecule into two or more simpler molecules. To illustrate, the following examples may be cited: If some yellow oxide of mercury is heated in a test-tube it is decomposed into metallic mercury and oxygen. If a proteid is exposed to the action of pure boiling sulphuric acid in a so-called Kjeldahl flask this very complex body is decomposed into carbon dioxide, water, ammonia, and other simple molecules. What is accomplished with the proteid by artificial chemical manipulation is

done under natural conditions, though much more slowly, by bacteria. Some of the latter, principally strict or facultative anaërobics, break down or decompose the proteid material into still comparatively complex molecules with the production of a fetid smell. This process is called *putrefaction.* Other bacteria, mostly aërobics, produce a more complete decomposition of the proteids into very simple compounds, like carbon dioxide, water, and ammonia. This process is known as *decay.* There is no generic difference between the processes of putrefaction and decay, but only one of degree. Some of the most common bacteria of putrefaction and decay have already been described, such as the Bacillus proteus, the Bacillus subtilis, the Bacillus mesentericus vulgatus, etc. The ammonia derived from decomposing urea and proteids may be formed in such a manner as to remain in the soil, or it may escape into the air, from which it is subsequently washed down into the soil with the rain. Plants, as stated, can utilize ammonia salts only to a slight extent for providing for their nitrogen requirements, and, hence, nitrogen in this form is of little value to higher vegetables which require nitrogen in the form of nitrates. Some bacteria have the power to ferment urea and change it into salts of ammonia; other bacteria possess the property of oxidizing ammonia salts into nitrates; this process is called *nitrification.* The oxidation of ammonia salts into *nitrates,* however, does not occur immediately, but through the intermediary process of the formation of *nitrites,* that is, salts of nitrous acid, which represents a lower stage of oxidation than nitric acid.

FERMENTATION OF UREA.

Quite a number of bacteria possess the property of fermenting urea and decomposing it into ammonia, from which salts are formed when the opportunity offers for the base to unite with an acid. It is probable that this converting power depends upon an enzyme known as *urase.* Some of the principal urea-fermenting bacteria are the following:

Micrococcus Ureæ.—This is a globular bacterium from 0.8 to 1 micron in diameter, frequently found in diplococcus or tetrad form. According to Leube it forms on *gelatin plates* after twenty-four hours, white cultures of the size of a millet seed, which have a mother-of-pearl luster, a sharp margin, and a smooth surface. After ten days the colonies are quite large and resemble somewhat a drop of stearin which has fallen upon and solidified on a surface. The growth does not liquefy gelatin.

Micrococcus Ureæ Liquefaciens.—This is a larger organism than the preceding. The cocci have a diameter from 1.25 to 2 micra. They appear singly or in chains of three to ten individual cocci. On *gelatin plates* the organism, after two days, forms in the depth small, white

points, which under a low magnification appear dark gray, round, with sharp margins. After growing up toward the surface the colonies become larger, assume a yellowish-brown color and a dark centre and slowly liquefy the medium. In *gelatin stick cultures* a white, confluent mass is first formed along the stick canal. It soon leads to liquefaction and the latter progresses until finally one-half or more of the medium has become liquefied, while the bottom is covered with a white, yellowish sediment.

Both the cocci described ferment urea when present in the culture media, and the decomposition continues until 13 per cent. carbonate of ammonium has been formed. The optimum temperature of development is between 30° to 33° C. The best artificial culture medium, according to von Jaksch, is one containing: Urea, 3 gr.; tartrate of sodium and potassium, 5 gr.; potassium monophosphate, 0.12 gr.; magnesium sulphate, 0.06 gr., and water in sufficient quantity to make 1000 c.c.

Urobacillus Pasteuri.—Miguel has isolated from air, soil, sewage, and water 60 different bacteria, all of which possess the property of fermenting urea. Of these 60 he has studied 17 species more particularly, and he distinguishes 3 types, namely, *the urobacillus*, the *urococcus*, and the *urosarcina*. The organism which has the greatest urea fermenting power was called Urobacillus Pasteuri by Miguel, who found it frequently in sewage. This bacillus can split up 140 grams of urea in 1000 c.c. of bouillon.

NITRIFYING BACTERIA.

The nitrifying bacteria possess very peculiar properties differing greatly from those of the bacteria considered in the preceding chapters. They do not require organic material for their growth and multiplication, and, in fact, do not grow properly in its presence in artificial culture media. The latter must contain simple chemical compounds only. Nitrifying bacteria do not require the presence of light in their synthetic metabolic processes. Nitrification, however, will take place in the soil in the presence of small amounts of organic matter, but any larger amount will stop it even in the soil, and it does not occur in the manure as first existing in a concentrated form. After putrefaction and decay in manure has decomposed most of its organic matter, nitrification can occur. The process, likewise, does not take place in an acid medium, and for this reason soils that have become quite acid by the decomposition of a large amount of organic matter must first be neutralized by carbonate of lime before there can be any progress in nitrification. The nitrifying bacteria have, however, a wide range of temperature, and nitrification occurs under otherwise favorable conditions between 37° F. to 110° F.; it is best at 99° F., and almost ceases at 122° F. Since nitrification is a process of oxidation, it requires the presence of considerable quantities of air, and the

more broken up and mingled with air the soil the better the process of oxidation of the ammonia salts.

The chemical action of these bacteria in the soil has been known for a long time, but the greater part of more accurate knowledge is due to Winogradsky, who was the first to devise methods of obtaining them in pure cultures. Winogradsky's first method consisted in the use of a silicon-jelly (water-glass jelly), difficult to prepare. The formulæ for his later fluid culture media, which are easier to prepare, were given in Chapter X. The pure cultures obtained enabled Winogradsky to show that the nitrifying bacteria consist of two groups, one oxidizes ammonia compounds into nitrites, the other group changes nitrites into nitrates.

Nitrosomonas and Nitrosococcus.—These are the bacteria of the first group. Their oxidizing action takes place according to the chemical formula $(NH_4)_2O + 3O_2 = N_2O_3 + 4H_2O$. *Nitrosomonas Europea* is found in soil in Europe. It is a short rod 1.2 to 1.8 micra long, provided with a short flagellum, and lively motile. *Nitrosomonas Javanica* was isolated from soil in Batavia, it is apparently round and coccus-like, has a diameter of 0.5 to 0.6 micron, and has a very long flagellum (up to 30 micra). *Nitrosomonas Japonica* and *N. Africana* are like the European variety. *Nitrosococcus* has been found in South America and Australia. They are large cocci, 1.5 to 1.7 micra in diameter, not motile, and possess no flagella. The organisms of this group, according to Winogradsky, are easily perishable when desiccated. The flagellate nitrosomonas in young cultures swarm around in a lively manner and impart to the medium an opalescent character; later they sink to the bottom, unite in *zoögleal masses*, and form a grayish, gelatinous, cloudy sediment. The best method to recognize the finer details of the structure of these organisms consists in treating cover-glass preparations with Gram's iodine solution. All varieties of nitrosomonas do not show the swarming stages, some form zoögleal masses from the start, and remain in this stage permanently.

Nitrobacteria.—These form nitrates from nitrites, according to the formula $N_2O_3 + 2O = N_2O_5$. They are small, oval, or pear-shaped bodies, 0.5 micron long, 0.15 to 0.25 micron wide. They form in fluid media a thin, shiny pellicle, firmly adherent to the vessel wall.

The two groups of nitrifying bacteria in soil act with such harmony that it is, as a rule, impossible to find any nitrites; nitrates alone can be discovered.

DENITRIFYING BACTERIA.

While the nitrifying bacteria have the power to oxidize nitrogen compounds like ammonia to lesser or higher stages of oxidization, there are other bacteria which have the property of reducing oxygen containing nitrogen compounds into lower stages of oxidation or even to take up all their oxygen and set the nitrogen free. Such organisms are called denitrifying bacteria in a general sense. Properly, however,

this term should be reserved exclusively for those microörganisms which are able to set nitrogen free from nitrites or nitrates. Many bacteria, particularly in the absence of free oxygen, under anaërobic conditions, can obtain their oxygen supply by a reduction of nitrates into nitrites, and among these is the Bacillus coli communis, the typhoid bacillus, and the Bacillus ramosus, or root bacillus. The bacilli which in the more strict sense are dentrifying bacteria, *i. e.*, those which can carry the reduction far enough along to the setting free of nitrogen, have been designated as *Bacillus denitrificans* α and β (of Gayon and Dupetit). Aberson's *Bacillus denitrificans* is a particularly energetic denitrifier and can reduce nitrates contained in pure cultures to complete liberation of all of the nitrogen. In addition to these three species, others of the group have been described by various investigators. These bacteria and other denitrifiers may cause great loss to the plant-available nitrates in soil under faulty methods of fertilization, as, for example, particularly when nitrates and fresh manure (especially horse manure) are distributed simultaneously to the soil.

FIXATION OF FREE NITROGEN.

The enormous quantity of free nitrogen contained in the atmosphere was formerly believed to be entirely useless in so far as assimilation by living organisms was concerned. Nitrogen is a comparatively inert gaseous elementary body, unlike oxygen, which easily enters into combination with a variety of other chemical elements and compounds. It has, however, been known for some time that certain plants can indirectly derive their nitrogen supply from the atmosphere through the intervention of bacteria. Most plants, if brought into a nitrogen-free soil, wither and perish after a short time; the *leguminosæ*, to which clovers, peas, beans, lentils, and similar plants belong, however, can thrive even in a nitrogen-free soil. They develop *nodules* from the size of a pea to that of a hazelnut on their roots. These root nodules of the leguminosæ contain innumerable bacteria which have united with the higher plant in a symbiotic community and which assimilate free nitrogen, transforming it in such a manner that it becomes soluble and available to the leguminous plant for the preparation of the required vegetable proteids. The exact details of this chemical transformation, however, are not fully known, but the nitrogen fixation by the bacteria of the root nodules of the leguminosæ is an established fact. Wronin was the first investigator who observed that the root nodules contained cells filled with bacteria. The microscopic examination of the nodule shows an outer, colorless cortical zone and an inner, pale red, later greenish-gray, medullary zone, which has rather irregular outlines and in shape somewhat resembles a mulberry. This inner zone is composed of the cells which contain the bacteria. Beyerinck, in 1888, first isolated such bacteria in pure cultures, and he named the organism isolated *Bacillus radicicola*.

Cultural Properties and Development of Nodule Bacteria.—Prazmowski recommends the following culture medium: An infusion is prepared in hot water from leaves of leguminosæ. After filtration and boiling, gelatin, 7 per cent.; asparagin, ¼ per cent.; saccharose, 0.5 per cent., are added. The medium is standardized so that it contains 0.6 c.c. normal acid to each 100 c.c The medium is kept in Petri dishes which are inoculated in the following manner: A young nodule is first washed in sterile water, afterward placed for a short time in strong alcohol, and finally washed in ether, which is allowed to evaporate. When dry the nodule is divided with a sterile knife and the juice which escapes is spread with a platinum loop over the surface of the medium, as it is there that the development of nodule bacteria occurs best. Beyerinck described the organism so obtained as showing two morphologic types. One is a rod 4 to 5 micra long, 1 micron thick, and the other a very small swarming rod, 0.9 micron long and only 0.18 micron thick. The small bacilli can pass the Chamberland filter and they wander away from their colonies on the soft gelatin plates to form new colonies at a distance. The larger rods are by no means regularly cylindrical, but some show branched forms shaped like a Y. The Bacillus radicicola does not liquefy gelatin, starch, or cellulose, and does not form spores. It is killed at 60° to 70° C., but is resistant to desiccation or freezing.

Role of the Nodule Bacteria.—The nodule bacteria of leguminosæ evidently differ, because those of one species often cannot form nodules in another species, and, as a rule, can infect only species which are very nearly allied. For instance, bacteria of the pea can form nodules of the root of the bean, but not on the roots of different species of clover; similarly, the bacteria of clover cannot infect and enter into symbiotic community with the families Vicia (pea) and Phaseolus (bean). *Conn* (Agricultural Bacteriology), discussing the role of these microörganisms, states: "It is practically certain that nearly all soils contain bacteria capable of living in symbiosis with leguminous plants. Nearly all soils except extremely sandy soils, that support little or no vegetation, will support leguminous plants and develop tubercles on their roots. One can scarcely examine the roots of legumes anywhere without finding tubercles, a fact which shows that the bacteria in question are very widely distributed in nature. But are the bacteria all of the same species? A very large number of species of legumes with their tubercles can grow in most, if not in all, soils. Are the bacteria that form tubercles on the clover the same as those which form them upon the pea, or is there a different species of bacteria for the different species of legume? It would not seem probable that there could be in the soil a different variety of bacteria for every variety of legume, but rather that one kind of bacteria can grow in many legumes. But the facts are not quite so simple as this. Not all species of legumes are capable of developing root tubercles equally well in all soils. Some soils will luxuriantly

support certain species of beans, peas, or clovers producing a large crop, developing quantities of tubercles and fixing an abundance of nitrogen, while the same soil will not support other species of legumes with equal readiness. . . . It certainly means that different species of legumes demand different varieties of tubercle bacteria. Whether these different varieties are distinct species is, of course, a fruitless question, inasmuch as we do not know what we mean by a species among bacteria. But it is of importance to know whether these types are quite distinct or whether they are simply physiological varieties of the same general species. If the former is true we should expect them to remain distinct, but if the latter is true, we might expect the soil bacteria to be capable of adaptation by cultivation to different legumes. On the whole, the evidence is decidedly in favor of the latter view and indicates that the different tubercle bacteria are probably all one general species, but that under different conditions they assume slightly different physiological relations. They can accommodate themselves to grow in one or another legume, and having become especially adapted for one species, but allowed to develop in the soil in which the latter plants are growing, they will adapt themselves in time to the new plant. In other words, experiments indicate that there is probably one species of tubercle bacteria, and that this species assumes different physiological characters under the influence of the different conditions in which it grows."

The Bacillus or Bacterium radicicola, according to Prazmowski, penetrates into the epidermis cells of the root hairs of leguminosæ and develops a colony which surrounds itself with a tough membrane. From the point of entrance a sac filled with bacteria is then formed. It grows toward the cortical cells and penetrates into the interior of the root hair, where it stimulates the cells to proliferation. The sacs, also called the *infectious filaments*, are not part of the leguminous plant, but are derived from the gelatinous (zoögleal) substance of the bacteria. The different parts which constitute the infected cells can be demonstrated by a mixed watery solution of fuchsin and gentian violet in 1 per cent. acetic acid. Sections of the nodules or tubercles stained with this solution show the plasmatic contents and the membrane of the leguminosa cells blue, the bacteria red, and their common zoögleal envelope and membrane unstained. The entire mass of the tubercles, composed of the infected leguminosa cells and the infecting bacteria, has been called the *bacterioid tissue*. The bacteria themselves, after infecting the higher plant cells, undergo changes and degenerate into involution forms which have been called *bacterioids* (this means bacteria-like forms). These are quite pleomorphous, have branches and sub-branches, and often form a more or less regular complete reticulum, or network. The nitrogen content of the dry substance of these root nodules of leguminosæ is very high. It was estimated by Stoklasa, at the time of flowering

of the plants, at 5.2 per cent.; at the time when the fruit begins to form, at 2.6 per cent.; and after the fruit had become ripe, at 1.7 per cent. The exact manner in which the nitrogen is taken up by the plant from the root tubercles is not known. Frank found even higher percentages—namely, 6.94 and 7.44 per cent. of nitrogen—and since the latter is present in the form of proteids it means a percentage of 43.4 and 46.5 of dry proteid substance.

Clover and other leguminosæ are now frequently used as so-called *green manure* for improving impoverished soil. These leguminosæ which can obtain their nitrogen supply with the assistance of the nitrogen-fixing bacteria from the air are planted in the nitrogen-poor oil. They are allowed to grow, and instead of being harvested are plowed under. The chemical and biological facts of nitrogen absorption have been known for a few decades only, but the ancient Romans had already noted the fact that impoverished soil could be improved by the planting of clover.

Clostridium Pasteurianum.—In addition to those bacteria which have the power of nitrogen fixation in symbiotic community with leguminosæ, there are other bacteria which exhibit the same power in soil alone and without being in symbiosis with other higher organisms. The investigations of Winogradski have demonstrated this. He described a nitrogen-fixing bacterium under the name of *Clostridium Pasteurianum.* It is a rod about 5 micra long, 1.2 micra wide, which produces end spores, and in doing so assumes the clostridium shape. At the same time it forms in its interior (but not at the poles) substances which are stained deep black blue with iodin solution. The mature spores escape in the longitudinal axis of the organism. The organism belongs to the group of *butyric-acid bacteria,* to which the bacilli of black-leg and malignant edema also belong. The Clostridium Pasteurianum is a strict anaërobe like most members of this group. It forms butyric and also acetic acid in the presence of carbohydrates, which are used as a source of energy; it can fix free nitrogen from the air. The organism does not grow on the general artificial culture media, but on *potatoes.* As the medium best adapted for its growth the following is recommended:

Phosphate of potassium (K_2PO_4)	1.0
Sulphate of magnesium	0.5
Chloride of sodium, Sulphate of iron, Sulphate of manganese	each 0.01 to 0.02
Carbonate of calcium, enough to neutralize.	
Glucose	20.00 to 40.0
Water, enough to make	1000.00 c.c.

As the Clostridium Pasteurianum is strictly anaërobic it can develop in soil only in the presence of aërobic bacteria, which use up the oxygen.

Azotobacters.—A group of aërobic bacteria able to fix nitrogen has been discovered by Beyerinck. He has named them *Azotobacter.* These organisms are oval bacteria, 4 to 6 micra long; they are

motile and possess flagella. Spore formation has not been observed. The *Azotobacter agilis* is more lively motile than the *Azotobacter chroöcoccum.*

QUESTIONS.

1. What is meant by the nitrogen cycle? Why is it necessary to preserve animal and vegetable life on our planet?
2. For what purpose do plants and animals need nitrogen?
3. In what form can plants utilize nitrogen for their nitrogen metabolism?
4. In what form do man and the lower animals mainly excrete the waste nitrogen?
5. What is meant by a metabolic waste product?
6. What becomes of urea excreted by animals?
7. What is meant by decomposition of complex chemical compounds?
8. Give some examples.
9. What is the difference between putrefaction and decay?
10. Name some putrefactive bacteria?
11. What is meant by nitrifying bacteria?
12. Describe some of their peculiar biologic properties.
13. Under what conditions will nitrification, brought about by bacteria, go on in the soil?
14. What microörganisms have the power to decompose urea? Name some of the most important ones.
15. Describe the Micrococcus ureæ.
16. Describe the Micrococcus ureæ liquefaciens.
17. Describe the Urobacillus Pasteuri.
18. What is the difference in action between the two groups of nitrifying bacteria?
19. Describe the different species of nitrosomas.
20. Describe a species of nitrosococcus.
21. In what fluid are they best examined microscopically?
22. Describe the morphology and the growth of nitrobacteria.
23. What is meant by denitrification?
24. Name some bacteria which in the absence of free oxygen can act as denitrifiers.
25. Under what circumstances can these bacteria produce great loss of nitrates in improper fertilization of the soil?
26. What plants have the power to utilize nitrogen from the atmosphere for their metabolism?
27. What are the root tubercles of leguminosæ?
28. What is the name of the bacillus contained in the root tubercles? What is its physiologic function?
29. What is the best culture medium for this bacillus? How can we obtain pure cultures of the Bacillus radicicola?
30. Describe the morphology of this bacillus.
31. Discuss the question whether or not there are a number of species of the Bacillus radicicola.
32. Describe how this organism penetrates into the root hair and what changes it produces.
33. What is meant by bacterioid tissue? Describe it in detail.
34. Discuss the nitrogen content of root tubercles at various periods of the plant's life.
35. What is meant by green manuring? What is its object and effect?
36. Name some bacteria which are nitrogen fixers but not in symbiotic community with higher plants.
37. Describe the morphologic and biologic properties of Clostridium Pasteurianum.
38. What kind of organism is azotobacter?

CHAPTER XLIV.

ACETIC-ACID BACTERIA.

NITRIFICATION consists in an oxidation of nitrogen, hence the nitrifying bacteria are oxidizing microörganisms. Bacterial oxidations are not confined to changes in the soil which are of the greatest significance to agriculture, but they play an important role in nature in the cycle of certain elements and in certain industries. One of these is the essential chemical process in the manufacture of vinegar, consisting in the conversion of alcohol into acetic acid. Alcohol formation from sugar is generally due to yeast cells (blastomycetes or budding fungi), and the conversion of liquids, such as beer, wine, or cider, containing alcohol into vinegar, that is, into a fluid containing acetic acid, is due to bacterial microörganisms. When alcohol containing liquids in open vessels and exposed to the air are changed into vinegar a tenacious slimy membrane is formed on the surface. It was noted long ago that pieces of this membrane rapidly changed alcoholic liquids into vinegar in the presence of free air; the membrane, therefore, received the name *mother of vinegar* (mere du vinaigre, French; Essigmütter, German). Persoon, in 1822, examined such membranes in various fermentative processes, and he called them *mycoderma,* which means a fungus, or slimy skin or membrane, but he did not believe in any causal connection between them and the fermentative process. Kuetzing, a German botanist, however, in 1832, declared that the mother of vinegar consisted of exceedingly small, punctate algæ, and that the conversion of alcohol into acetic acid was due to their metabolism. Kuetzing's claims never attracted much attention, and had long been forgotten, when more than forty years later Pasteur, in his studies on fermentations, again maintained that the conversion of alcohol into acetic acid was due to the life activity of microörganisms which he called *Mycoderma aceti.* Pasteur, however, did not exhaustively study the morphology of these vinegar organisms; this was later done by Hansen, who distinguished three different species of bacteria as the cause of the acetic-acid formation from alcohol. These he named Bacterium aceti, Bacterium Pasteurianum, and Bacterium Kuetzingianum. The three species are found in beer which is not very rich in alcohol and which is undergoing acetic-acid fermentation. Bacterium aceti forms a moist, slimy, smooth pellicle with fine lines; Bacterium Pasteurianum, a rather dry pellicle; and Bacterium Kuetzingianum one which resembles that of Bacterium aceti, but is much thicker, elevated, and reaches up along

the sides of the vessel. The pellicles formed are zoögleal masses in which the individual bacteria are held together by a plasmatic, gelatinous substance. In the case of Bacterium aceti the latter is not stained by iodine solution, while in the other two vinegar bacilli it is stained blue. The bacterial protoplasm proper of all three species stain yellow with iodin solution. Another acetic-acid bacterium, commonly known in England as vinegar plant, has been described by Brown as the Bacterium xylinum. It forms tough, leathery, thick zoögleal masses. The three species of Hansen also show marked differences in pure cultures on solid media (wort gelatin). Bacterium aceti forms very delicate, rosette-like colonies; Bacterium Pasteurianum develops colonies with a smooth, round periphery and folded surface, and Bacterium Kuetzingianum shows smooth colonies without any surface folds. All three types are short, rather thick bacilli, but they exhibit considerable pleomorphism under various conditions. Their optimum temperature of growth lies between 34° and 42° C., and they cease multiplication between 5° and 7° C. When raised at a temperature of 34° C. the Bacterium Pasteurianum forms chains of bacilli which are about 2 micra long and 1 micron thick. If these are transferred to a fresh medium kept at 40.5° C., long filaments are formed in which no dividing lines can be seen, and which reach a size of from 40 to 200 micra. If these are again transplanted to a fresh medium kept at 34° C., globular or elliptical swellings are formed in the threads, and these later break up into short rods and pear-shaped or globular cells, sometimes measuring 10 micra in diameter. The other two acetic-acid bacilli of Hansen under similar conditions likewise show much pleomorphism and even develop branched forms.

The conversion of alcohol into acetic acid is a process of oxidation, represented by the formula:

$$\underset{\text{Alcohol.}}{C_2H_6O} + \underset{\text{Oxygen:}}{O_2} = \underset{\text{Acetic acid.}}{C_2H_4O_2} + \underset{\text{Water.}}{H_2O}.$$

In the manufacture of vinegar from alcoholic liquids, such as wine, beer, and cider, provision must always be made that the air has free access to the fermenting fluid. This is accomplished by either allowing air to enter the barrels from one side and to escape from another, or by leading the fermenting fluid through barrels, vats, or tubes containing masses of wood shavings. This arrangement spreads the fluid over a large surface and allows it to mix thoroughly with air. Pure cultures of acetic-acid bacteria are not yet generally used in the manufacture of vinegar, though it appears that there would be considerable advantage in such a procedure. The use of pure cultures of yeasts, as is well known, has done very much in improving the quality of beer and preventing losses from the development of undesirable, so-called wild yeasts

QUESTIONS.

1. What kind of chemical process is the conversion of alcoholic liquids into vinegar?
2. What is vinegar?
3. What generally causes the conversion of glucose into alcohol and carbon dioxide?
4. What causes the conversion of alcohol into acetic acid and water?
5. What is meant by the mother of vinegar?
6. What was the first scientific name proposed for mother of vinegar?
7. Name some acetic-acid microörganisms.
8. Describe their morphology and their cultural characters.
9. How do the zoöglea of different acetic-acid bacteria behave toward iodin solution?
10. What is the chemical equation for the conversion of alcohol into acetic acid and water? What kind of a process is it?
11. What is the general arrangement of the manufacture of vinegar?

CHAPTER XLV.

THE BACTERIOLOGY AND THE BACTERIOLOGIC EXAMINATION OF MILK[1]—GENERAL INTRODUCTORY CONSIDERATIONS—THE CHANGE OF LACTOSE INTO LACTIC ACID—LACTIC-ACID BACTERIA—ANAËROBIC BUTYRIC-ACID FORMERS—PEPTONIZING BACTERIA—ALCOHOLIC FERMENTATION OF MILK.

GENERAL INTRODUCTORY CONSIDERATIONS.

While milk is an excellent culture medium for many bacteria, there are also many which do not find in milk conditions favorable to their growth. It is practically impossible to obtain milk from animals in an absolutely germ-free or sterile condition. This statement can perhaps best be explained by the analogy of surgeons' attempts to obtain completely germ-free hands before an operation. Such endeavors were begun soon after the knowledge of pyogenic wound infection microörganisms and their ubiquitous nature became established and have been continued for several decades. A vast literature upon this subject has accumulated, but notwithstanding trials continued for many minutes up to one-half hour and more it is now conceded to be impossible to sterilize the human hands. While washing and scrubbing with disinfectants, such as soap and water, dilute alcohol and corrosive sublimate solution may render the surface of the hands temporarily germ free, the cracks and recesses of the skin and the ducts of the sweat and sebaceous glands remain infected with bacteria, which when the hands are used and when perspiration occurs soon find their way to the surface.

This being the case it would be unreasonable to expect that the external surface of the udder and the hands of the milker could be so sterilized as to render them entirely free from bacteria. Even were this possible the bacteria always occurring in the milk-ducts still remain, and they would find their way into the milk during milking. For this reason it may be stated that milk after collection is practically

[1] The bacteriology and hygiene of milk have been treated as fully as is consistent in a text-book on bacteriology. For a more extensive consideration the reader is referred to the following works: Sommerfeld, Handbuch der Milchkunde, Wiesbaden, 1909; Jensen, Essentials of Milk Hygiene, translated by Pearson, Philadelphia, 1909; Conn, Bacteria in Milk, Philadelphia, 1903; Swithinbank and Newman, Bacteriology of Milk, New York, 1903; Milk and its Relation to Public Health, Public Health and Marine Hospital Service of the United States, Washington, Bulletin No. 56; Russel, Outlines of Dairy Bacteriology; Ward, Pure Milk and the Public Health; Winslow, Clean Milk.

never sterile, nor did nature attempt or intend to furnish to the young animal or infant an absolutely sterile germ-free food supply. The milk as drawn by suction from the milk glands becomes mixed with bacteria from the ducts, and the skin and the bacterial contents are further much augmented, before the stomach is reached, by the admixture with the secretions of the mouth, which contain very numerous bacteria.

The general statement that absolutely sterile milk can never be obtained refers, of course, to the practical collection of the fluid from the cow. A trained bacteriologist, after the liberal removal of the foremilk and a larger amount of milk flowing subsequently, can collect a few cubic centimeters of bacteria-free milk in a sterile test-tube, as has been shown in Bergey's extensive bacterial milk analyses.

What has been said, however, must not be taken to imply that efforts should not be made to obtain milk in such a way as to keep its bacterial contents as low as possible. This can best be accomplished by using the greatest care in collecting the milk, by cleaning the udder, cleansing the milker's hands, tying the tail of the cow during milking, receiving the milk into a clean (if possible sterile) vessel washed out with boiling hot water, protecting the lacteal fluid afterward from contamination with dust and dirt, and cooling it rapidly and keeping it cool to prevent subsequent bacterial growth and multiplication.

Numerically, most bacteria in contaminated milk are derived from fecal matter of the cow. Wüthrich and Freudenreich have ascertained that the feces of the cow contain about 375,000,000 bacteria per gram of moist substance, hence it is of the greatest importance in collecting milk to guard against contamination with cow's dung and against the dust and dirt derived from it. In practice, however, it has been recognized that a certain amount of dust and dirt contamination is unavoidable, and various bacteria from those sources, in addition to those habitually present in the milk-ducts, must be expected to be found, no matter how clean and ideal the environments of the milch cow's stable. Renk and others have elaborated methods to ascertain the amount of dirt which can be removed by sedimentation from milk, and which can subsequently by exact chemical methods be dried and weighed. European authorities appear to agree that milk collected by cleanly methods should contain less than 10 milligrams of dry dirt per liter, *i. e.*, less than one part to 100,000 parts of milk; but otherwise good market milk often contains an amount many times in excess of this standard.

THE CHANGE OF LACTOSE INTO LACTIC ACID.

Even when milk is collected in a very clean and careful manner it will undergo certain changes and generally become sour unless permanently kept at a temperature very near the freezing point of

water. This souring of milk is due to the accumulation of lactic acid, which is formed from the *lactose*, or milk sugar, of the milk. This change is brought about by a great variety of bacteria, which, broadly speaking, are always present in milk, and which, in their relation to milk, are known under the collective name of *lactic-acid bacteria.* The grouping is entirely arbitrary and artificial, because the organisms belong to various types and have in common only the property of splitting up lactose and forming from it lactic acid.

The change of lactose into lactic acid is chemically a process of *hydrolysis, i. e.,* a chemical change in which water is added to a molecule, and this molecule subsequently becomes split up into other compounds. The change is probably due to a so-called soluble ferment or enzyme, secreted by the lactic acid bacteria or contained in their bodies, where it may act upon the lactose which diffuses into the substance of the bacterium by osmotic processes. The chemistry of the process is expressed by the following formula:

$C_{12}H_{22}O_{11}$	+	H_2O	=	$2C_6H_{12}O_6$	=	$4C_3H_6O_3$
Lactose	+	Water	=	1 Glucose and 1 Galactose	=	4 Lactic acid

In order to understand the names given to some of the bacteria of the lactic-acid group it is necessary to know that lactic acid is a *stereoisomeric* body. This term means a body or chemical substance existing in nature in two forms of crystallization, which bear the relation to each other of a physical object to its image in a plane mirror and which, when in solution, will behave in the following peculiar manner toward rays of polarized light: One form of crystals, or bodies in solution, will deflect or deviate the polarized rays of light from their straight path toward the right side, and these are called *dextrogyr;* the other form will deviate or deflect the polarized rays of light from their straight path toward the left side, and these are called *sinistrogyr,* or *levogyr.* By a mixture of these two forms a solution may be obtained which will deflect the polarized light, neither to the right nor to the left side.

THE LACTIC-ACID BACTERIA.

The bacteria most commonly found in milk and producing in it the most rapid and obvious changes are those which possess the power to ferment milk sugar (lactose) and form from it lactic acid. They are known collectively as the lactic-acid bacteria and occur very widespread in nature. They have been found in hay and straw (Leichmann, Gruber, and others), also in the dust in barns and other places, on ordinary grasses and cereals, and other cultivated plants. Beyerinck found them in the feces of man and animals, and Barthel has found them practically wherever man, animals, and cultivated

plants are found. It has already been stated that numerically the most important source of bacteria in milk is cow's dung, in which enormous numbers of bacilli of the colon-aërogenes group are found. These are lactic-acid formers. Such bacteria, however, may also enter the milk from other sources. It was pointed out in the chapter on the Bacilli of the Colon Group that a number of investigators look upon the Bacillus coli communis as a ubiquitous organism. If this is the case its presence in milk cannot be taken as an absolute indication of fecal contamination. Rodgers and Ayers, in Circular 135 of the Bureau of Animal Industry, state that in some middle Western States organisms of the colon aërogenes group are commonly found on grass, grain, and in slough holes, and that, therefore, these lactic-acid and gas formers may not be derived from a fecal source when cows are milked in open fields. The discovery that the lactic-acid fermentation of milk is due to bacteria was first made by Pasteur and later confirmed by Lister. Hüppe gave the first description of a bacterium of this type and named it Bacillus acidi lactici, and originally believed that the latter was the only microbe which caused lactic-acid fermentation of milk. It was, however, soon shown by a number of investigators (Grotenfeld, Marpmann, Conn, Weigmann, and others) that a great variety of bacteria occur in milk which possess the power to ferment lactose. Some of them produce a dextrogyr, others a sinistrogyr lactic acid. The former type was found first. To the second class belongs Leichmann's Bacillus lactis acidi and Micrococcus acidi levolactici and Kozai's Bacillus acidi levolactici.

Classification.—The lactic-acid bacteria are now, according to Loehnis, as quoted by Weigmann, divided into four groups, namely:

Group I.—Plump, Gram-negative, gas-forming rods designated as Bacterium pneumoniæ of Friedländer or Bacterium acidi lactici of Hüppe.

Group II.—Elongated, oval, or lancet-shaped, Gram-positive streptococci, growing anaërobically and forming very little gas designated as Streptococcus pyogenes or, better, as Streptococcus Guentheri. This group contains the most important lactic-acid formers occurring in milk.

Group III.—Long, slender, Gram-positive bacilli, growing better anaërobically and forming little gas, designated as Bacterium caucasicum or Bacterium casei.

Group IV.—Gram-positive staphylococci, aërobic, forming no gas, generally liquefying gelatin and designated as Micrococcus pyogenes or Micrococcus lactis acidi.

Bacteria of Group I.—The bacteria of the first group are generally short rods, 1 to 1½ micra long and 0.75 to 1 micron thick. They occur singly or in groups of two, and also in the form of longer chains They are not motile and do not form spores, generally they are Gram negative. They are aërobic and facultative anaërobic. As a rule, they coagulate milk in from one to two days, sometimes later,

and occasionally coagulation does not occur at all, but the milk becomes thready or slimy. The lactic acid formed is generally of the sinistrogyr variety. These bacteria grow in milk between 15° and 45° C., and best between 30° and 40° C. They often impart a disagreeable taste to milk and occasionally milk containing them in very large numbers causes vomiting. These bacteria ferment other sugars, in addition to lactose, and then form, besides lactic acid, also succinic, acetic, and some formic acid; they also sometimes form alcohol and carbon dioxide and hydrogen. The several types in this first group are represented as follows:

Type 1.—Gas formers: Bacillus acidi lactici Hüppe, Bacillus lactis aërogenes Escherich, Grotenfeld's Bacillus acidi lactici, the fan-bacillus of Clauss, and Lustig's typhoid-like bacillus.

Type 2.—These coagulate milk but do not form gas: Bacterium limbatum of Marpmann, Bacillus sputigenes of Pansini and several others.

Type 3.—No coagulation, but formation of gas. The principal representative of this type is the Bacillus pneumoniæ of Friedländer.

Type 4.—This is represented by Bacillus lactis inocuus of Wilde, Bacillus No. 14 of Conn, Bacterium cocciform of Migula, and others. Neither coagulation nor gas is produced.

Bacillus Lactis Viscosus.—There are several other types of organisms in the first group of lactic-acid producers which make milk slimy or which liquefy the casein subsequent to its coagulation. Many bacteria of the first group are so intimately related to the colon bacillus that a separation from it becomes impossible. The most important organism of the kind which make milk slimy or ropy is the Bacillus lactis viscosus of Adametz. The organism has been isolated from water which probably is its normal habitat and from which it gets into milk. Ward has always found this organism in slimy milk. It grows at very low temperatures, better than at higher temperatures, and for this reason its multiplication is favored by the rapid cooling of milk. It possesses a gelatinous, slimy capsule and forms zoögleal masses; it is this property which imparts to milk the slimy, ropy character. Such milk is probably not unwholesome, but it is repulsive and disliked by consumers.

Bacteria of Group II.—The second group, known as the streptococcus group, comprises cocci which are not motile, form no spores, are either round, semiglobular or oval and form shorter or longer chains. They are Gram positive and frequently show a capsule. There are pathogenic and non-pathogenic bacteria in this group, the former growing best at 37° C., the latter at 30° to 35° C. They develop both aërobically and anaërobically. They generally form much lactic acid and coagulate milk; sometimes both processes occur slowly. Coagulation is sometimes brought about by an enzyme of the rennet type without the formation of acid. The lactic acid formed is generally of the dextrogyr variety. These bacteria also

split up other sugars, but they generally form lactic acid only, rarely other acids. This group again contains a number of types distinguished and represented as follows:

Type 1.—Streptococcus mastiditis coagulates milk and forms gas.

Type 2.—Streptococcus Guentheri or Leichmann and Streptococcus lacticus Kruse both coagulate milk, but do not form gas. *These two organisms are probably the most common and most important lactic acid bacteria of milk.*

Type 3.—Streptococcus Kefir, does not coagulate milk and does not form gas.

Type 4.—Streptococcus lactis inocuus, does not coagulate and does not form gas.

Type 5.—Leuconstoc mesentericus and Micrococcus mucilaginosus of Schütz and Ratz, which make milk slimy.

Type 6.—Streptococcus mirabilis Roscoe, which is an arborescent organism.

Type 7.—Streptococcus coli gracilis and coli brevis, which are liquefiers.

Bacteria of Group III.—The bacteria of the third group vary much in length. They are most commonly slender rods, 2 to 3 micra long and 0.5 to 0.75 micron wide. Some of them form filaments or pseudofilaments 50 or more micra long. They are generally not motile, never form spores, rarely show a capsule, and are Gram positive. They are either preferably or even strictly anaërobic. Their optimum temperature is generally quite high, between 40° and 50° C., their minimum at 25° C. or somewhat lower. Milk is generally coagulated very slowly; the lactic acid formed is generally sinistrogyr. None of them are disease producers. They are divided into types, the most important of which are:

Type 1.—Bacillus casei of Freudenberg.

Type 2.—Bacterium casei of Leichmann.

Type 3.—Bacterium caucasicum.

Type 4.—Bacillus Delbrücki.

Type 5.—Bacillus Aderholdi.

Type 6.—Bacillus lactis acidi Leichmann.

There are no liquefying bacteria represented in this group.

Bacteria of Group IV.—The bacteria of the fourth group are of the type of the Micrococcus pyogenes Rosenbach or Micrococcus lactis acidi. They are cocci varying in size from 0.8 to 1.6 micron. They are single, in pairs, or in irregular groups (staphylococci). They do not form spores, are Gram positive, have their optimum of growth between 20° to 30° C., and multiply best in the presence of oxygen. Some of them liquefy gelatin, others do not. Most of them coagulate milk. Formation of gas is rare. The types in this group are represented by the following bacteria:

Type 1.—Micrococcus acidi Leischmann.

Type 2.—Micrococcus lactis acidi Marpmann.

Type 3.—Micrococcus butyri aromafaciens.
Type 4.—Micrococcus candicans Flügge.
Type 5.—Micrococcus lactis viscosi Gruber (causing slimy or ropy milk).
Type 6.—Micrococcus coronatus Flügge.
Type 7.—Micrococcus cirrhiformis Migula (forms gas).

The Coli Aërogenes Bacteria.—Under the preceding four collective groups of lactic-acid bacteria proper a number of microörganisms have been mentioned. Those named, however, are only a small fraction of the bacteria of the groups which now comprise several hundred, though it is very probable that many which in fact are identical species have been described by different observers under different names. Besides these lactic-acid bacteria *par excellence*, others, such as the Bacillus coli communis and the Bacillus lactis aërogenes of Escherich, form lactic acid from sugar of milk. The Bacillus coli communis has been fully described in a previous chapter as evidently including a number of varieties.

The Bacillus aërogenes or the Bacterium lactis aërogenes of Escherich is generally plumper and shorter than the Bacillus coli communis. It is 1 to 2 micra long and 0.5 to 1 micron wide; it presents itself singly, in pairs, rarely in chains, or pseudofilaments. It is not motile, does not form spores, and possesses no flagella. On gelatin it forms large, white, not transparent colonies. It splits glucose with the formation of gas. It also ferments lactose in milk, but forms from it more acetic than lactic acid. It also forms succinic acid. Such lactic-acid bacteria proper as the Streptococcus Guentheri and Streptococcus lacticus in their growth in milk largely prevent the development of the bacteria of the coli aërogenes group.

ANAËROBIC BUTYRIC-ACID FORMERS.

Anaërobic bacteria which, in addition to lactic acid, also form butyric acid from lactose are frequently found in milk. The first bacterium of this group was discovered by Pasteur, who also recognized its anaërobic nature. Prazmowski first introduced the name of Clostridium butyricum for a bacterium of this group; later a number, several of which were evidently identical, were described under various names. Beyerinck united the butyric-acid bacteria into a family under the name of *granulobacter*, or *amylobacter*, because these organisms when growing in a medium containing starch form characteristic granules in their interior. A number of very important anaërobic pathogenic bacteria belong to this family, such as the bacillus of black leg, the bacillus of malignant edema, and others described in previous chapters.

Schattenfroh and Grassberger have classified the butyric-acid formers into four groups, as follows:

1. Motile butyric-acid bacteria.
2. Gas formers of the black-leg bacillus type. (*a*) Spore-forming (black-leg bacillus, Bacillus aërogenes capsulatus); (*b*) non-motile (non-pathogenic) butyric-acid bacilli.
3. Organisms of the type of the malignant edema bacillus.
4. Putrefying butyric-acid bacilli of the type of Bacillus putrificus Bienenstock.

The motile butyric-acid bacillus is relatively prevalent as a saprophyte in soil, water, grain, flour, cheese, more rarely in milk, which generally does not form a favorite soil for its development. It grows best, according to Beyerinck, in artificial culture in a 5 per cent. *peptone solution* (under anaërobic conditions). It is a long, slender rod, motile, and with flagella surrounding the entire body. Before sporulation the bacillus forms granulose[1] from starch in its interior and assumes the clostridium shape. The *spore* escapes from its membrane generally at one end, and the empty shell may for some time remain over one end of the young bacillus like a cap. The spores are killed when exposed in boiling water for three minutes to 100° C. On *gelatin* the organism forms cloudy, hazy colonies; the colonies may also be better defined and surrounded by filamentous excrescences or they may form a veil on the surface of the medium without any distinctly defined boundaries whatever. The bacillus, while fermenting dextrose, saccharose, lactose, starch, and glycerin, and forming from them butyric acid, lactic acid, carbon dioxide, and hydrogen, does not split up albumin. Butyric acid is formed in excess of lactic acid and hydrogen in excess of carbon dioxide. In *milk* a floating layer of casein full of gas-bubbles is formed. This motile butyric-acid bacillus is identical with the bacilli described under the following names: Bacillus amylobacter I and II, Gruber, Granulobacter saccharobutyricus Beyerinck, and Bacillus saccharobutyricus of Klecki.

The non-motile butyric-acid bacillus is likewise frequently found in nature, and as it is a regular inhabitant of the feces of cattle, often in milk. According to Botkin-Rodella it can be easily obtained by inoculating sterile milk covered by a cream layer 10 cm. high from garden earth. The milk, while still heated to 70° C., is inoculated and then kept in the incubator, when the non-motile bacillus generally grows abundantly. The organism occurs in two types. The first type presents cylindrical rods, with rounded ends, generally forming chains of three to six links, or pseudofilaments 20 to 50 micra long. Its colonies are small, very shining, and surrounded by numerous excrescences. The second type forms shorter and more slender rods, rarely arranged in longer chains, and its colonies are round, sharply defined, smooth, and dewdrop like. Both types in starch-agar form

[1] Granulose formed from starch in the interior of the bacilli of this group like starch stains blue with iodin solution.

granulose in their interior and spores. The latter can resist boiling in water at 100° C. for one and one-half hours. These organisms liquefy *gelatin* and coagulate *milk*. Grassberger claims that the non-motile butyric acid bacilli are black-leg bacilli without pathogenic properties.

The Bacillus putrificus Bienenstock is a slender rod, 5 to 6 micra long, with rounded ends. In *gelatin*, which is liquefied, it forms long chains and pseudofilaments. The spores are formed at one end like those of the tetanus bacillus and the sporulating organism has the drum-stick appearance. This is best shown on *agar* or *blood-serum* cultures. The spores can withstand heating at 80° C. for three minutes, but they are killed in boiling water in five minutes. On *agar slants* kept under anaërobic conditions the organism forms a transparent veil and clouds the mass of the medium, also the water of condensation. The organism may be obtained from hard cheese, ground up fine and incubated with a *nutrient bouillon*, containing 0.5 c.c. lactic acid, kept under anaërobic conditions in the incubator for ten to fourteen days.

Other butyric-acid bacteria which have frequently been found in milk are the Bacillus lactopropylbutyricus of Tissier and Gashing, the Clostridium Pastorianum Winogradsky, and the Clostridium Americanum of Pringsheim. The latter is not as strictly anaërobic as the other species enumerated. This organism was found on American potatoes.

A bacterium frequently found in soft, strong-smelling cheese is the Paraplectrum fetidum of Weigmann. It is a rod from 2.5 to 8 micra long, 0.6 micron thick; it forms spores in milk in two to three days, and coagulates this fluid in forty-eight hours. The coagulation is soon followed by a peptonizing liquefaction, with the formation of a very fetid smell similar to that of limburger cheese.

PEPTONIZING BACTERIA IN MILK.

There are two groups of spore-forming, aërobic bacteria in particular which are very widespread in nature. They secrete enzymes and almost invariably get into milk, in which they coagulate the casein and subsequently liquefy it again. The coagulation, brought about by a rennet enzyme, may be only very slightly marked, because the peptonizing ferment is furnished by these bacteria so promptly and evidently so abundantly that the peptonizing and liquefying action greatly overshadows the coagulation. The bacteria of this type are characterized by two representative species, the Bacillus subtilis, or hay bacillus, and the Bacillus mesentericus vulgatus, or common potato bacillus. They are present in the soil, in manure, on grains, potatoes, hay, and straw, and in air and water. They are putrefying organisms which, in connection with anaërobes with whom they live in symbiotic community, are engaged in breaking up organic waste

material into very simple chemical compounds. They get into milk during milking, with the dust from hay and straw, and they are undoubtedly also found on the skin of the cow's udder. They are not numerous in cow's feces, hence they do not indicate contamination by manure.

Bacillus Subtilis.—The Bacillus subtilis can be easily obtained by making an infusion of hay, boiling it for one hour, and incubating it at 37° C. The boiling destroys the vegetative forms of all kinds of bacteria, but does not affect the spores of the Bacillus subtilis. The latter is a rod 4 to 5 micra long and 0.8 to 1.2 micron thick; sometimes very short rods are seen, also long chains and pseudofilaments. The bacillus is motile and possesses eight to twelve flagella, arranged around the body of the organism. The spores are formed in the centre, sometimes more toward one end of the bacillus. When germinating they rupture the spore membrane in the equatorial plane, not at either end. The elongating young bacillus sometimes carries the empty spore membrane along, attached to it like a cap. On *gelatin* small, white, punctate colonies are formed, which later become darker, granular and brownish, and send out hair-like processes into the culture soil. The colonies rapidly liquefy gelatin. On gelatin stick cultures rapid liquefaction occurs along the whole line of the stick. On *agar slants* a heavy, corrugated growth is formed. *Blood serum* is likewise liquefied. The bacillus grows well and abundantly on *potatoes*. As already stated, it peptonizes *milk* very rapidly, but its growth in milk is prevented as soon as lactic-acid bacteria have formed a small amount of lactic acid. According to Lafar the presence of 0.1 per cent. of lactic acid prevents the development of the Bacillus subtilis. It is a strictly aërobic organism, and requires for its best growth an abundant supply of oxygen. The organism is Gram positive.

Bacillus Mesentericus Vulgatus.—The Bacillus mesentericus vulgatus is a widely prevalent saprophyte. It is the most common of the group of potato bacilli and is somewhat smaller than the Bacillus subtilis. It is motile and possesses numerous flagella, which generally are found on one side of the body only. The spores are elliptical and large. On *gelatin* the growth closely resembles that of the Bacillus subtilis and liquefies it energetically. In *milk* coagulation is more marked than in the case of the subtilis, but subsequent liquefaction is also soon accomplished. On *potatoes* the growth is very abundant and forms a thick, corrugated layer. The organism occurs in several varieties, such as the Bacillus mesentericus fuscus, which forms a grayish-brown pigment, and the Bacillus mesentericus ruber, which forms a reddish-yellow pigment. The Bacillus liodermes, or rubber bacillus of Loeffler, which also belongs to the same group, forms a rubber-like growth on potatoes.

The common root bacillus, Bacillus mycoides, so called on account of its rizoid cultures on gelatin, is also frequently found in milk.

Other Peptonizing Bacteria.—A number of other organisms frequently found in milk, which coagulate the casein and subsequently peptonize or liquefy it, have been described by Duclaux. These are the Tyrothrix or Bacillus tenuis, Tyrothrix or Bacillus distortus, Tyrothrix or Bacillus geniculatus and several others of the same family. These organisms are aërobic and facultative anaërobic, spore forming, motile or immobile rods. Tyrothrix geniculatus peptonizes the casein without preceding precipitation.

It is generally believed that the bacteria of the Bacillus subtilis group play an important role in the ripening of cheese; also the different species of tyrothrix described by Duclaux.

CHROMOGENIC BACTERIA IN MILK.

Fluorescent and chromogenic bacteria sometimes impart a particular color to milk. The former kind sometimes produce a green fluorescent pigment. The Bacillus cyanogenus, a motile, Gram-negative, non-sporogenous bacterium, produces a bluish to brownish-red pigment. The Bacillus violaceus, a water bacterium, sometimes gets into milk and produces a violet color, as does also the Bacillus membranaceus amethystinus. Sarcina rosacea stains cream pinkish, while the Bacillus prodigiosus may produce in cream a more decidedly red color. Bacillus lactorubefaciens stains the whole milk red and makes it slimy, Bacillus mycoides roseus gives it a rust-brown color, Bacterium synxanthum stains it yellow.

ALCOHOLIC FERMENTATION OF MILK.

Under certain conditions yeast cells, or saccharomyces, are found in milk. They possess the faculty of forming alcohol from lactose. The enzyme which brings about this fermentation is called *lactase*, and is not identical with the yeast enzyme known as zymase, which splits monosaccharids into alcohol and carbon dioxide. Most of the microörganisms which form alcohol from lactose are not true yeast cells, or saccharomyces, but belong to the nearly related family known as *Torula*. The principal generic difference between yeast cells and torula is that the former form spores, but the latter are *asporogenous*. The most important organisms which form alcohol in milk from milk sugar are the following:

Saccharomyces lactis acidi of Grotenfeld acidulates milk, coagulates it, and forms some alcohol; Saccharomyces Freudenreich and Jensen, Saccharomyces fragilis of Joergensen, and Torula lactis of Adametz. The latter is a torula which was discovered by Weigmann. It forms in milk from lactose 51.2 per cent. by weight of alcohol, 34.4 per cent. of carbon dioxide and 3.6 per cent.of butyric acid. The so-called

kefir or *kafyr granules*, which are used in the Orient to prepare an alcoholic beverage from milk, contain both bacteria and yeast cells. The cause of the fermentation of the alcoholic beverage, known as *kumys*, prepared in Siberia from mare's milk and ass's milk, is not known.

In addition to the bacteria, saccharomyces and torula, higher moulds (*i. e.*, those forming a true mycelium) are likewise found in milk. Among such moulds may be mentioned Oidium lactis, Penicillium glaucum, Penicillium roqueforti, Penicillium camemberti, and Mucor racemosus. Many of these moulds play an important role in the ripening and flavoring of cheese.

QUESTIONS.

1. Is it possible in practice to obtain germ-free milk from the cow in larger amounts? If not, why not?
2. What measures are necessary to keep the bacterial content of milk as low as possible?
3. What is the most important source of increase of the bacterial contents of milk.?
4. What should be the maximum of dirt permissible in milk?
5. What brings about the souring of milk? What change occurs in lactose or sugar of milk in the process of souring?
6. What is meant by a stereo-isomeric body?
7. What is meant by dextrogyr lactic acid? What is meant by sinistrogyr lactic acid?
8. What is polarized light?
9. Where are the lactic acid bacteria found?
10. Describe in general terms the four groups of lactic-acid bacteria.
11. Name some of the various types belonging to each of the four groups.
12. What are the morphologic features of the most common types of lactic acid bacteria?
13. What is meant by the coli-aërogenes bacteria?
14. What are the anaërobic bacteria found in milk?
15. What are the four groups of butyric acid formers?
16. What is a clostridium?
17. Describe some of the butyric-acid formers.
18. What peptonizing bacteria commonly occur in milk?
19. Describe the Bacillus subtilis.
20. Describe the Bacillus mesentericus vulgatus.
21. Name and describe some of the chromogenic bacteria found in milk.
22. Name some organisms producing alcohol in milk.

CHAPTER XLVI.

THE BACTERIOLOGY AND THE BACTERIOLOGIC EXAMINATION OF MILK (CONTINUED)—PATHOGENIC BACTERIA IN MILK—THE TUBERCLE BACILLUS—METHODS FOR DETERMINING ITS PRESENCE IN MILK—HUMAN AND OTHER CATTLE DISEASES TRANSMISSIBLE THROUGH MILK—NUMBER AND SIGNIFICANCE OF LEUKOCYTES IN MILK.

PATHOGENIC BACTERIA IN MILK.

THE organisms, such as the lactic-acid bacteria, the butyric-acid bacteria, the alcohol formers, and the higher moulds, which have already been discussed, are saprophytes and not disease producers. Pathogenic bacteria, however, also occur in milk. They may be derived directly from the cow, from those handling the milk, or they may be accidentally introduced with water or otherwise.

Tubercle Bacillus.—Of the microörganisms pathogenic to man which may occur in milk, tubercle bacilli are the most important. It is possible to keep disease-producing bacteria, such as the typhoid, diphtheria, and other bacilli out of milk by having the proper persons collect it properly in sterile vessels; but tubercle bacilli which come from the cow cannot, under certain conditions, be kept out of the milk. This is undoubtedly the case in tuberculosis of the udder itself and in advanced general tuberculosis. Examinations of market milk for the presence of tubercle bacilli have been made in many cities and the positive findings vary greatly. Anderson, in 223 specimens of milk examined in Washington, D. C., found 15 (6.27 per cent.) with live, virulent, tubercle bacilli; Hess, in New York, in 106 samples found 17 (16 to 17 per cent.); Delepine, in Manchester, in 125 samples found 22 (17.6 per cent.); Eber, in Leipzig, in 210 samples found 22 (10.5 per cent.); Klein, in London, in 100 samples found 7; and Petri, in Berlin, in 86 samples found 33 (38.4 per cent.). Very low figures are reported from Italy where in a number of cities no tubercle bacilli were found in any of the samples examined; also from Würtemberg, where Herbert examined 101 samples without any positive findings. The highest findings have been reported by Kanthack and Sladen, who found 9 samples out of 16 infected in England, and by Obermüller, in Berlin, who took 14 samples from one place and found them all infected. Schroeder states that most tubercle bacilli get into milk from fecal contamination. He reports that he has been able to show in a number of experiments their presence in the feces of cows which

were in good physical condition and in which the diagnosis of tuberculosis could be made only by the tuberculin test. He also calls attention to the fact that tubercle bacilli may not be voided continually by such animals, and that they may occur in the milk from certain dairies and sources only on a few days during several weeks. Eber has made similar observations in regard to the intermittent distribution of tubercular milk by dairies in Leipzig. Schroeder seems to believe that tubercle bacilli may be excreted with the feces of tubercular cows and otherwise, even in the absence of open tubercular lesions, but this view is certainly not shared by many. At the Eighth International Congress in Washington, Bang stated that when the tubercular lesions in a cow are closed no tubercle bacilli are present in either the milk or the feces. When some of the lesions are open and the bacilli enter into the lymph or blood circulation the bacilli may be temporarily present in the milk or feces. Heymans believes that Schroeder's results with the inoculations of feces into guinea-pigs are not quite conclusive as to the fecal excretion of tubercle bacilli in an early stage of tuberculosis in cattle.

In considering the significance of bovine tubercle bacilli in milk it must be borne in mind that the presence of a small or even a larger number of bacilli sufficient to produce tuberculosis in a guinea-pig by intraperitoneal or subcutaneous injection[1] *may*, but generally *will not*, produce tuberculosis in man, even when ingested as food for a considerable period. That this is indeed the case has been repeatedly shown by a number of observers, and the following investigations are of interest:

Hess, of the Research Laboratory of the Health Department of New York City, obtained specimens of raw milk in New York from large forty-quart cans. He collected this milk from dealers who had small children who drank the milk in a raw state. He secured 107 specimens, and in 17 of these tubercle bacilli able to kill guinea-pigs were found; 8 stems were subsequently obtained from the dead animals and 7 were found to be of the bovine and 1 of the human type. This induced the author to point out that tubercle bacilli in milk might occasionally be derived from man and not from cattle. Of the dealers who had dispensed the milk containing tubercle bacilli, ten were found to have 18 children who had been regularly fed on this raw milk; 9 of the children were two years or under and only 1 was over five years. The children were carefully watched for one year, and 16 were submitted to the Pirquet tuberculin test; 4 reacted in a positive manner; of the 4, 2 were perfectly healthy and 2 were poorly nourished, but showed no definite signs of tuberculosis. Of the 2 in poor condition, 1 had five months previously had

[1] Knutt has shown that a tubercle-bacilli-containing-milk which would produce tuberculosis in guinea-pigs in intraperitoneal inoculations of 0.00001 gram (*i. e.*, a dose of $\frac{1}{100}$ of a milligram) would only produce tuberculosis if fed in a dose of 15 grams, *i. e.*, one million and a half (1,500,000) times the intraperitoneal dose.

a cervical adenitis.[1] From the fact that almost all of the children who had been regularly drinking infected milk were found in good health, the author concluded that milk containing bovine tubercle bacilli does not necessarily or even usually excite tuberculosis in children.

On account of the extent and importance of the discussion of the transmissibility of bovine tuberculosis to man, the German Imperial Health Office instituted a collective investigation of a number of cases in which milk from cows suffering with tuberculosis of the udder was regularly and for a long period of time consumed raw. Kossel has recently published a summary of the results of the investigation. It was found that 360 persons had consumed such milk; of these 200 were adults, 151 children, age not given for 9. Two children aged one year and ten months, and one year and three months, respectively, showed undoubted evidences of infection with bovine tuberculosis.

In both the cervical glands were affected, and in the younger child tubercle bacilli of the bovine type could be demonstrated. In examinations, however, respectively one and one-half and two and one-half years subsequent to the first examination both children showed a healthy development and a good appearance, notwithstanding the fact that they had been fed on raw milk from cows with advanced udder tuberculosis. Four adults and 8 other children which had habitually consumed the same raw milk remained well. In 12 cases of the 360, tuberculous infection was suspected, but examination made months and years after the first did not reveal any evidence of glandular tuberculosis; in fact, the gland swellings had disappeared and some of the physicians who had made the earlier examinations doubted the correctness of their former diagnosis. In reviewing the results of this investigation, Weber concludes that the danger to man from raw milk from tubercular cows is very small in comparison with the danger from persons with open pulmonary tuberculosis, and he agrees with Flügge and Osterman's claim that great numbers of tubercle bacilli must be present before much danger of infection exists.

Methods for the Determination of the Presence of Live Tubercle Bacilli in Milk.—As milk and milk products, such as cream and butter, frequently contain acid-fast saprophytes which cannot morphologically be distinguished from the tubercle bacillus, the usual staining method used for exhibiting this organism can only be considered a preliminary procedure, and the finding of acid-fast bacilli must be followed by a proper inoculation of the suspected material. The steps in the complete examination are the following:

I. Centrifuge the milk in an electric centrifuge for five to ten minutes. A layer of cream is then formed on top and a sediment at the bottom of the tube.

[1] It was ascertained in a subsequent examination that the tubercle bacilli responsible for the cervical adenitis in this child were of the typus humanus. (Personal communication from Doctor W. H. Park, Director of the New York Health Research Laboratory.)

II. Remove carefully with a small sterile spoon (it is best to use a platinum spoon) the fat from the top and place it in a sterile covered watch-glass or Petri dish.

III. Pour off the fluid layer forming the middle stratum, then likewise, preserve the sediment in a sterile vessel.

IV. The cream and the sediment may be mixed or examined separately. In very exact examinations it is best to inoculate a mixture of cream and sediment of each sample of milk into a guinea-pig or several of these animals. This is rarely done, as it requires too many experimental animals and too much really unnecessary labor.

As a rule, several cover-glasses are prepared from the mixed creams and sediments, and they are examined by the Ziehl-Neelson method of staining with carbol-fuchsin solution. The samples which contain acid-fast bacilli are diluted with sterile physiologic salt solution and inoculated subcutaneously into guinea-pigs, best in the neighborhood of some lymph gland (inguinal glands) with the suspected material. In experiments for the detection of live tubercle bacilli in milk, butter, etc., the inoculations should always be made subcutaneously, not intraperitoneally, because in the latter case guinea-pigs generally develop pseudotuberculous lesions in the presence of the acid-fast bacilli of Möller, Rabinowitsch and others, even when tubercle bacilli are absent.

For the microscopic examination of butter for acid-fast bacilli the following procedure is recommended by Roth:

I. Two to 4 grams of butter are placed in a sterile centrifuge tube, which is filled about three-quarters full with sterile, distilled water.

II. This tube so prepared is placed in a water bath and kept at 50° C. until all of the butter is melted.

III. The tube is now closed with a cork or glass stopper and well shaken for some time to mix the fat and water thoroughly. It is now placed into the incubator in an inverted position, *i. e.*, with the cork or glass stopper down and the pointed end of the centrifuge tube up. After all the fat has risen to what is now the upper end, the tube is left in a cool place until the butter fat has again solidified.

IV. The tube is now inverted, the cork or stopper opened, the wash water which now contains most of the bacteria is poured into another sterile centrifuge tube, and this is again centrifuged.

V. The sediment is then used for cover-glass preparations, and these, when air dry, are washed for a short time in an absolute alcohol-ether mixture.

VI. The cover-glass preparations are then stained and decolorized in the usual manner.

Typhoid Bacillus.—The bacilli which cause the human disease typhoid fever are voided in enormous numbers during and after the course of the affection with the feces. By getting into wells supplying farms and dairies from vaults, cesspools, and other sources, or by

contaminating ponds, brooks, and rivers they may pollute water, and by this means the disease is generally spread to human beings. It has also been ascertained that certain persons, after having passed through an attack of typhoid fever may harbor numerous typhoid bacilli in their intestines for years without injury to themselves and discharge them in their stools. Such persons who have been called "permanent carriers" may become a source of great danger to their surroundings or to the water supply of a larger territory.

Most typhoid epidemics are waterborne, but epidemiological statistics have also shown that a high percentage of typhoid epidemics, preferably those in a limited territory, are milkborne. Typhoid bacilli generally find their way into milk through contaminated water. Such water being used cold to wash out milk cans and a sufficient quantity may remain in the container to bring about infection, as milk is a favorable culture medium for typhoid bacilli. When typhoid bacilli get into raw milk there is first a marked decrease in their number, but later a great increase which, however, does not lead to such changes as acid formation and coagulation, which might betray its abnormal character. If, however, the lactic-acid bacteria have greatly increased in number and have produced a marked acidity typhoid bacilli are first inhibited in their growth and later killed off entirely. Bassenge states that a degree of acidity of 0.3 to 0.4 per cent. in milk will kill typhoid bacilli after twenty-four hours. They survive, however, for a long time in fresh, raw milk which has been infected and is kept at a low temperature. The bacilli perish rapidly in sour cream, but they may survive for a considerable time in sweet cream and in butter. Schüder, who investigated 638 smaller and larger epidemics of typhoid fever and 12 individual cases, came to the conclusion that the disease was due to water 462 times (70.8 per cent.) and to milk 111 times (17.0 per cent.). Trask, in analyzing 179 typhoid epidemics, chiefly in the United States and England, as spread by milk, found that 113 were traceable to farms, dairies, or milk shops. These conclusions, however, rest solely upon epidemiological data, because typhoid bacilli have only very rarely been found in milk. Vaughan reported the finding of typhoid bacilli in milk in 1890, but at that time the bacilli were not indubitably identified; in 1906, however, Konradi isolated bacilli from milk which were fully identified by agglutination and other tests; Shoemaker also obtained typhoid bacilli from milk, handled by a person convalescent from typhoid fever. Flies have undoubtedly conveyed typhoid bacilli from feces to milk. Levy and Jacobstal claim to have isolated typhoid bacilli from a large abscess in the spleen of a cow. According to the most trustworthy information, domestic and other animals are not susceptible to natural infection with typhoid bacilli, and if the case just mentioned was indeed one of typhoid it must have been a very exceptional occurrence.

Whether paratyphoid bacilli are spread by milk is a still unsettled

question, as our knowledge of small epidemics due to this micro-organism is still too fragmentary.

Cholera Spirilla.—Three small epidemics of Asiatic cholera, apparently traceable to contamination of a milk supply, have been reported by Gaffky, Simpson, and Knüppel; the spirillum of Asiatic cholera itself has never, however, been found in milk.

Dysentery Bacilli.—Dysentery bacilli of the Shiga, Flexner, Kruse, or Strong type, which have been isolated in cases of human dysentery in adults and children, have never been found in milk; but since epidemics have been traced to water, the possibility of the occasional presence of these bacilli in milk cannot absolutely be denied.

Bacillus Diphtheriæ.—The Bacillus diphtheriæ has been occasionally found in milk, and a number of small epidemics of diphtheritis have been traced to the milk supply. Dean and Todd found a case of particular interest in which 4 persons were infected by the milk of a certain cow suffering from teat ulcerations which contained diphtheria bacilli. The ulcerations, however, were not due to the diphtheria bacilli which were present in the open sores only as an accidental contamination. Trask, in analyzing the literature, found 23 diphtheria epidemics due to milk, 15 in the United States and 8 in Great Britain. Such milk epidemics usually show a sudden, almost explosive onset, because the infected milk imparts the disease simultaneously to a number of persons. Milk is a favorable culture medium for the Bacillus diphtheriæ, which is introduced into it through coughing, sneezing, or otherwise, by persons engaged in collecting and handling milk, who have more or less recently passed through a severe, light, or entirely masked and undiagnosticated attack of the disease, and who still harbor the bacilli in the pharynx. Several milkborne diphtheria outbreaks in California have been reported by Ward.

Scarlet Fever.—Scarlet fever outbreaks, according to Trask, have been traced to milk 51 times. As the cause of scarlet fever is still unknown the statements as to milkborne epidemics are not very trustworthy, and it must be remembered that the virus may be spread by those who deliver the milk and in whom the affection may be so mild as not to be recognized as scarlet fever. Typical cases in children, for instance, not infrequently lead to sore throats in adults, and the latter, in whom the infection is not recognized, may spread the typical disease to others. The author has encountered such a case of *masked scarlatina* in a nurse who spread the disease to several persons. The true state of affairs, so far as the nurse herself was concerned, was only accidentally discovered by a complement deviation test.[1] Scarlet fever is, of course, not a disease of cattle, and McFadyean's experiments to produce the affection experimentally in calves were all negative.

[1] For complement fixation test see Chapter VII. This is the test used to discover latent syphilis in persons (Wassermann test), and it is sometimes also positive in scarlet fever.

CATTLE DISEASES TRANSMISSIBLE THROUGH MILK.

Foot-and-Mouth Disease.—In addition to tuberculosis a number of other diseases of cattle may be transmitted through the milk to man. Some of these affections are due to bacteria, others are due to a filterable, invisible living virus. Among the latter, foot-and-mouth disease is of importance. It is transmissible from afflicted animals to man through raw milk, buttermilk, butter, cheese, and whey. Man, however, does not seem very susceptible to the virus because in European countries the affection often occurs in widespread epidemics among cattle, yet cases in man are rare. The disease in man may take a light, a serious, or even a fatal form. According to Busenius and Siegel, 172 cases of hoof-and-mouth disease infection occurred in man from 1886 to 1896, of which 66 could be traced to milk and 1 to butter. A few cases transmitted through butter and 1 case conveyed by soft cheese were reported after 1896. It has also been proved by a number of experiments that the disease, after it has been transmitted to man can be retransferred to cattle.

Anthrax.—It has been shown by Bollinger, Feser, Nocard, McFadyean and others that the milk of cows suffering from anthrax may contain anthrax bacilli. Cows sick with anthrax, however, generally soon go dry; yet anthrax bacilli may occasionally be transferred through milk. Karlinski has reported the case of a patient convalescent from typhoid fever who drank a quart and a half of milk brought by a visitor. The patient shortly afterward became very sick again; anthrax bacilli were found in his feces, and he died after one month, as the postmortem showed, from the intestinal form of anthrax. It was also shown that the cow which had furnished the milk had, in the meantime, died from anthrax. Bonhoff, in the examination of 39 samples of butter, once accidentally found anthrax bacilli. These reports show that milk and milk products may occasionally, though very rarely, contain virulent anthrax bacilli.

Enteritis in Cows.—Bacilli of the colon-paratyphoid group, causing enteritis in cows, may occasionally lead to intestinal disturbances in man. Gaffky has reported a case where the milk of a cow suffering from hemorrhagic enteritis caused such disturbances in three persons. Klein observed an epidemic of diarrhea in London which he thought was due to the Bacillus enteritidis sporogenes infecting cow's milk from the feces of these animals, but Hewlett and Barton, who found this bacillus in 60 per cent. of the samples of London market milk examined for this reason consider Klein's claim as entirely unfounded.

Trembles and Malta Fever.—The transmission of trembles, or milk sickness, from cows to man and of Malta fever from goats to man have been discussed in Chapters XXXII and XXXIV.

Bacteria of Mastitis in Cows.—The bacteriology of inflammation and suppuration of the udder in cows (bovine mastitis) is quite fully

discussed in Kitt's contribution on this subject to Kolle and Wassermann's *Manual on Pathogenic Microörganisms.* L. Frank was the first investigator to hold that mastitis in cows was generally due to an infection, and, in 1875, he conducted experiments showing that the disease could be produced by injecting fluids containing certain bacteria into the udder, and that it could then be again transferred from diseased to healthy cows. The bacteriology of bovine mastitis was subsequently studied by a large number of authors, among whom may be mentioned Rivolta, Dickerhoff, Mollereau, Nocard, Kitt, Bang, Lucet, Guillebeau, Jensen, and others. The disease may have a very rapid onset (over night), and it may then become chronic or it may from the beginning have an insidious, slow, and chronic course. The milk, in cases of very acute onset, shows marked changes; it often contains coagula, which are sometimes stained by admixture with blood. In the slow, chronic cases not much change is evident in the lacteal fluid. Sometimes one-quarter of the udder alone is affected, at other times two, three, or all of the four ventricles. Sometimes mastitis leads to grave general symptoms, with high fever and prostration. Recovery may occur in ten to thirty days, or the disease may extend over weeks and months; it may make a cow dry and produce atrophy or necrosis of the udder.

The bacteria most commonly found in suppurative inflammations of the udder are: Bacilli of the colon group, first called Bacillus phlegmasia uberis by Kitt, now simply known as colon bacillus. The organisms of this group have already been fully described. Streptococci are frequently the cause of mastitis in cows, but their presence in milk is by no means conclusive evidence of an inflammation. In fact, some of the most common lactic-acid bacteria in milk are of the non-pathogenic streptococcus type. Heinemann, however, has succeeded, by repeated passages through the bodies of rabbits, in changing non-pathogenic streptococci from milk into a type pathogenic to rabbits in subcutaneous and intravenous injections. This, however, proves nothing as to any original pathogenicity when the organisms are taken into the stomach of man with milk. A large number of observers have frequently found streptococci in market milk, as for instance Bergey (Philadelphia, 50 per cent. of the samples), Eastes (England, 72.5 per cent), Bruening (Leipzig, 93 per cent.), Conn and Esten (Middleton, 100 per cent.) and many others. Most of the streptococci commonly found in milk belong to the type of Streptococcus lacticus, one of the common lactic-acid formers in milk, and these cannot by any known tests, except possibly occasionally by animal inoculations, be distinguished from the Streptococcus pyogenes. If it is known that a cow has mastitis, then the streptococcus present may be of the pathogenic, pyogenes type; but the disease of the udder may also be due to bacilli of the colon group and the streptococcus present be the non-pathogenic lacticus. The organism known as Streptococcus mastitidis vaccarum forms both short and

very long chains, and does not liquefy gelatin. Some of the stems isolated from cases of bovine mastitis form acid in sterile milk and coagulate it, others do not form acid, nor lead to coagulation. In considering the pathogenicity to man of streptococci found in cattle, it must be remembered that the streptococcus generally found as the cause of suppurative processes in cattle and known as the Streptococcus pyogenes bovis is not fully identical with the Streptococcus pyogenes found in man, from which it differs in certain cultural and other characteristics. (See chapter on Pyogenic Bacteria in Domestic Animals.)

Staphylococci have likewise been frequently found in inflammation of the udder of the cow, and Guillebeau has distinguished the following four types: Staphylococcus mastitidis, Galactococcus versicolor, Galactococcus flavus, and Galactococcus albus. Lucet has described five different staphylococci of bovine mastitis, but he has not proposed special names for them. Mastitis in the cow has been produced experimentally by the injections of Bacillus suipestifer, Streptococcus equi, Bacillus avisepticus, and Botryococcus ascoformans.

NUMBER AND SIGNIFICANCE OF LEUKOCYTES IN MILK.

Milk always contains certain cellular elements. The fluid secreted during the very first stage of lactation, called colostrum, shows corpuscles filled with fat granules known as *colostrum corpuscles.* The derivation of these bodies has been a contested question for a long time, but the preponderance of evidence now appears to be that the majority of them are mononuclear leukocytes, and only a small number are granular epithelial cells, both filled with fat granules. The colostrum corpuscles disappear soon after the beginning of lactation, and the most important cells always found in milk are the leukocytes, or white blood corpuscles. Several authors, among those who have studied the significance of the number of leukocytes found in milk under various conditions, have very seriously discussed the question how to distinguish in milk between leukocytes and pus corpuscles. This must appear somewhat futile in view of the fact that there is no generic difference between a leukocyte and a pus corpuscle. Histopathologic investigations have shown beyond doubt that a pus cell is nothing more or less than a leukocyte, which, in consequence of inflammatory stimuli and positive chemotactic influences, has wandered out of a bloodvessel into the perivascular tissue or into a preëxisting or pathologically formed cavity.[1] In inflammatory conditions of the mammary glands an increase in the number of leukocytes in milk must be expected. It appears, on first thought, that the leukocyte count would aid in the detection of milk derived from a diseased udder, and indicate whether or not it should

[1] This subject has been fully discussed in the Chapter on Phagocytosis.

be condemned as improper for use, repulsive, and probably unwholesome. The leukocyte contents of wholesome milk from healthy cows, however, varies so much as shown by more recent investigations based upon accurate methods, that older standards resting upon inaccurate methods and upon insufficient data must be given up.

Methods of Estimating Leukocytes in Milk.—STOKES' METHOD.—Place 10 c.c. of milk into a centrifuge tube and centrifuge in an electric or other good centrifuge for ten minutes. Draw off the fat and the clear fluid by the aid of a pipette and leave only the sediment in the tube. Spread a platinum loopful of the sediment over 1 square centimeter on a glass slide or cover-glass. Air dry, fix and stain with methylene blue; wash in water, dry between filter paper and mount. Count ten fields of a one-twelfth oil-immersion lens and calculate the average per field. It has been customary on the basis of this method to declare milk unfit for use when the number of leukocytes exceeded ten leukocytes per field.

STEWART'S METHOD.—Small glass tubes are used, closed at one end with a rubber stopper. Place 1 c.c. of milk into one of the tubes, centrifuge for ten minutes. Then hold the tube horizontally and draw out the rubber stopper, to which the sediment adheres. Spread the sediment over one square centimeter area of a glass slide or cover-glass. Air dry, fix and stain with methylene blue, and count the leukocytes in ten fields of a one-twelfth oil-immersion lens. Milk has been considered unfit for use if the average per microscopic one-twelfth oil-immersion field is above twenty-three.

TROMMSDORF'S METHOD.—In this method specially constructed centrifuge tubes are used holding 10 c.c. and drawn out at the bottom into a fine caliber. The narrow part of the tube has twenty graduations, each one representing 0.01 per cent. of 10 c.c. If the sediment, therefore, reaches to the tenth mark it indicates that the 10 c.c. of milk contain 0.1 per cent. sediment or one volume of the latter to 1000 of milk. According to Trommsdorf this is the maximum amount admissible, and anything above represents an excess in leukocytes, *i. e.*, pus. While this method simply indicates the percentage of sediment and gives no information as to its composition, it is said to be a fairly accurate and good practical method.

DOANE-BUCKLEY METHOD.—This is the most accurate, reliable procedure, because actual count of the leukocytes is made with a Thoma-Zeiss counting chamber (see below). The method, as modified by Campbell and described in Bulletin 117 of the Bureau of Animal Industry, includes the heating of the milk to be tested, as this separates the leukocytes from the fat globules, and gives higher and more accurate values. The effect of heating on the leukocyte count had been previously described by Russell and Hoffmann. The steps in the Doane-Buckley method are as follows:

1. In this as in any other method the sample examined should be as fresh as possible, because the formation of lactic acid favors the precipitation of casein, and this interferes with obtaining a clear

specimen. If milk must be brought from a distance to the laboratory it is advantageous to add a few drops of formalin to 100 c.c. of the milk to prevent changes in reaction. The milk should be well shaken, then 10 c.c. are filled into a centrifuge tube. The latter is now immersed in a water bath at 65° to 70° C. for ten minutes, or at 80° to 85° C. for one minute. The tube, while still warm, is at once centrifuged for ten minutes.

2. Draw off 5 or 6 c.c. of the fat and watery fluid, leaving the sediment undisturbed. Add enough warm distilled water to make up to 10 c.c. Shake well and centrifuge again. Repeat this procedure several times and a clear sediment free from fat is obtained. Finally, draw off all fluid except 1 c.c., which is left in the centrifuge tube. Shake well, place a small drop on a counting chamber, and count several hundred squares. Estimate the average number of leukocytes per square and from it calculate the number of leukocytes present in 1 cubic centimeter of milk. Ward recommends leaving only ½ c.c. in the centrifuge tube.

Thoma-Zeiss Counting Chamber.—This instrument, which is used in counting red and white blood corpuscles in blood, milk, pus, urine, and other fluid media, is constructed as follows: On the centre of a strong slide a small round glass plate of exact known thickness is mounted; a second glass plate, also of known thickness, with a larger circular opening in the centre is so mounted on the heavy slide that it is outside of the small round glass plate in the centre. The outer plate is exactly $\frac{1}{10}$ mm. higher than the small round inner plate, which is ruled in such a manner that 1 square millimeter has been divided into 400 equal squares. In other words, each one of the small ruled squares is equal to $\frac{1}{400}$ of one square millimeter. In using the counting chamber a small drop of fluid containing the corpuscles (in the present case milk sediment) is placed on top of the centre of the inner round (ruled) glass plate. A clean, rather thick cover-glass is then placed over the drop. This must be done very carefully in an inclined manner, to prevent air-bubbles from entering between the glass and the drop of fluid. The cover-glass, after being in place, is now (at the margin where it rests on the outer plate) pressed down with a tissue needle, glass rod, or lead pencil. Some of the fluid which is between the cover-glass and the small, central, ruled glass plate will run into the moat formed between the round inner and the outer plate. The whole slide is now lifted to the stage of the microscope and is focussed with a $\frac{1}{6}$ or $\frac{1}{5}$ inch objective.[1] A certain number of leukocytes, easily recognizable by their nuclei and more or less granular protoplasm are now seen. The number of leukocytes in 200 squares is counted. Every space seen through the microscope has a base of $\frac{1}{400}$ square millimeter and its height is $\frac{1}{10}$ mm., which is the distance of the cover-glass resting on the outer plate from the inner ruled,

[1] As the specimen is unstained the iris diaphragm must be closed so that the field is only dimly lighted.

small, round, glass plate. Each of the spaces seen in the field of the microscope represents accordingly $\frac{1}{4000}$ of a cubic millimeter.

If the four hundred squares ruled on the inner plate were alike and undivided into groups the eye would easily lose track and an accurate count would be difficult. For this reason the first one of each group of five squares both from right to left and from above downward has an additional ruling going through the centre of the square. These lines, however, are ignored in counting, and their only object is to enable the observer to see when he has counted the cells in five, ten, fifteen, or twenty squares, or any multiple of five squares.

Calculation of the Result.—Suppose that the sediment contained in 1 c.c. of what was left in the tube is taken and that 242 leukocytes have been counted in two hundred of the squares; this would mean an average of 1.21 leukocytes per square. Since each square represents $\frac{1}{4000}$ cubic millimeter, each cubic millimeter of the sediment would contain 1.21×4000, or 4840 leukocytes. In blood work it is customary to indicate the number of leukocytes present in *1 cubic millimeter* of blood; but in milk, *1 cubic centimeter* is used as the standard. Since 1 cubic centimeter is equal to 1000 cubic millimeters, it is necessary to multiply 4840 by 1000. This gives 4,840,000, which indicates the number of leukocytes present in the 10 c.c. of milk, as 1 c.c. of the sediment examined contained all of the leukocytes in the 10 c.c. originally used. To obtain the number of leukocytes in terms of 1 c.c. it is, therefore, necessary to divide 4,840,000 by 10, which gives 484,000. In other words, it is found that 242 leukocytes present in two hundred squares means that the milk examined contained 484,000 leukocytes per 1 c.c.

The simple mechanical rule for finding the number of leukocytes is therefore: After repeated centrifuging, leave enough of the clear fluid with the sediment to make the total of sediment and clear fluid equal to 1 c.c. Shake well; prepare counting chamber; count the number of leukocytes in 200 squares and multiply the sum obtained by 2000 $= (\frac{4000 \times 1000}{200 \times 10})$.

If only 0.5 c.c. was left in the centrifuge tube then the sum of leukocytes found in 200 squares is multiplied by 1000.

When the leukocytes are very numerous it is sufficient to count them in 100 squares only; when they are scanty it is better to count them in 400 squares. In the former case the sum must be multiplied by 4000; in the latter by 1000, in order to obtain the figures for 1 c.c. of milk.

Campbell has made numerous comparative tests, and he finds that heating the milk increases the leukocytes in the sediment no matter what method of sedimentation and estimation is employed. He also points out that more recent knowledge concerning the leukocyte contents of milk compels the dismissal of former standards as unreliable and inequitable.

Variability in the Leukocyte Count in Milk from Healthy Cows.—Russell and Hoffmann found that the milk of some of the cows

investigated was remarkably uniform, while in a considerable number of cases the results varied greatly. The more uniform results were found in cows with no previous history of udder trouble of any kind. Some cows of this kind, however, also showed great variations in the count. Eighteen animals in a perfectly healthy group showed in 537 tests a leukocyte contents of 500,000 or less in 90 per cent. of the cases; however, in 16 tests in this group over 1,000,000 were found. The authors state that it is apparent from their studies that the leukocyte content of normal milk drawn from apparently normal animals is quite often so high that the milk would be classed as coming from diseased animals when judged by the standards that have heretofore been proposed and that therefore complete reliance cannot be placed upon quantitative leukocyte standards alone. Stone and Sprague made 1167 leukocyte counts in two perfectly healthy cows during a period of about ten months, examining each day both the morning and the evening milk. These counts varied from 2,110,000 to 10,000. The highest counts of over 2,000,000 occurred the first two days of lactation, but counts between 100,000 and 500,000 were found in 29 per cent. of all counts. The authors state that their confidence in an arbitrary numerical leukocyte standard as the reliable criterion of the sanitary fitness of milk has been very much shaken; but they believe that the physiological average is considerably below 500,000. Ward, likewise, is of the opinion that a leukocyte count alone does not generally furnish data from which the presence or absence of inflammatory conditions in the udder can be diagnosticated.

QUESTIONS.

1. Discuss the presence of tubercle bacilli in milk. In what form of tuberculosis are they most frequently found in milk?
2. In what percentage of market milk have they been found?
3. Is every milk containing tubercle bacilli likely to produce tuberculosis in man?
4. What is the procedure for discovery whether samples of milk contain live virulent tubercle bacilli?
5. Give in detail the method of searching for tubercle bacilli in butter.
6. How can typhoid bacilli get into milk?
7. What is known about milkborne typhoid epidemics?
8. How may the diphtheria bacillus get into milk?
9. What is known about milkborne scarlet fever epidemics?
10. What other cattle diseases, aside from tuberculosis, may be transmitted through milk?
11. What are the most common bacteria in mastitis in cows?
12. Discuss the number and significance of leukocytes in milk?
13. What is a colostrum corpuscle? Where does it come from?
14. What is Stokes' method of estimating leukocytes in cow's milk?
15. What is Stewart's method?
16. What is Trommsdorf's method?
17. Which is the most accurate method of estimating leukocytes in milk? Describe its details.
18. Describe a Thoma-Zeiss blood-corpuscle counting chamber.
19. Describe the method of calculating the result of a leukocytic count in milk.
20. What effect has heating the milk upon the leukocyte count, and why?
21. Describe the variability of the leukocyte count in the milk of normal cows.

CHAPTER XLVII.

THE BACTERIOLOGY AND THE BACTERIOLOGIC EXAMINATION OF MILK (CONTINUED)—QUANTITATIVE ESTIMATION OF BACTERIA IN MILK—INTERPRETATION OF THE RESULTS OF BACTERIAL COUNTS IN MILK—DETERMINATION OF THE ACIDITY OF MILK—CERTIFIED MILK—PASTEURIZATION OF MILK—ITS ADVANTAGES AND DISADVANTAGES—STORCH'S TEST.

QUANTITATIVE ESTIMATION OF BACTERIA IN MILK.

THE investigations of von Freudenreich, Henderson, and others have shown that the milk ducts in the udder of perfectly healthy cows contain numerous bacteria. Sedgwick and Batchelder, MacConkey, Burr, von Freudenreich, Lux, and others have found the bacteria contents of milk freshly drawn under all possible aseptic precautions, and after the removal of the foremilk[1] and an additional liberal amount of regular milk to be from 250 to 1500 per cubic centimeter. But these figures form no basis for practical deductions when a large amount of milk is collected in one pail. Russell found that the mixed milk from a good herd, collected with care and cleanliness, examined immediately after milking, showed from 5000 to 20,000 bacteria per cubic centimeter. After milk has been collected the number of bacteria apparently decreases for a number of hours. This is attributed to the germicidal property of milk. This question has recently been re-investigated by Rosenau and McCoy, who come to the following conclusions:

"Judged by the number of colonies that develop upon agar plates, the bacteria in milk first diminish, then increase in numbers. This so-called germicidal property of milk occurs only in fresh, raw fluid. For the most part our work plainly shows that no actual reduction in the number of bacteria occurs. However, when compared with the controls, a restraining action is evident. The phenomenon, therefore, appears to resemble that of a weak antiseptic rather than that of a true germicide. When milk is kept warm (37° C.) the decrease is pronounced within the first eight or ten hours. After this time the milk has entirely lost its restraining action. When the milk is kept cool (15° C.) the decrease is less marked, but more prolonged. The decrease in the number of bacteria is largely apparent, being due,

[1] L. Schulz found in various specimens of foremilk obtained from healthy udders, after thorough external disinfection, from 50,000 to 79,000 germs per cubic centimeter.

at least in part, to agglutination. The germicidal action of milk is specific. This action in milk and blood serum resemble each other in some particulars, but blood serum acts more quickly and much more powerfully than milk. Heating milk above 80° C. destroys its germicidal properties. The effect of lesser degrees of heat varies with the microörganism. Thus the restraining action for Bacillus lactis aërogenes is weakened by first heating the milk at 55° C. and almost destroyed at 60° C."

While raw milk, therefore, shows a diminution of bacteria during the first hours after collection, a rapid multiplication occurs later, particularly if the milk has not at once been cooled and kept at a low temperature permanently until used for consumption. If this has been done the number of bacteria does not increase much for thirty-six hours, but milk, according to Park, contains many species of bacteria which will multiply even at 39° F., *i. e.*, at a temperature not much above the freezing point of water. Park found that a specimen of milk containing originally 3000 bacteria per cubic centimeter, kept at 32° F., showed a decrease of microörganisms after seven days; this was also true in a specimen containing originally 30,000 per cubic centimeter. At 39° F. the counts, after seven days, showed 4 and 38 million respectively; at 42° F., 11 and 120 million; at 50° F., after four days, 12 and 300 million, and at 60° F., after two days, 28 and 163 million. These figures and those ascertained by various observers, including Conn, Esten, Harrison, and others, demonstrate the great influence of higher temperatures upon bacterial multiplication in milk. Since milk shipped to cities by farmers and dairies is frequently insufficiently cooled, market milk often shows a high count even in samples which have been collected with reasonable care and which do not show much dirt contamination. Rosenau found in the milk of Washington, D. C., an average of 22,134,000 bacteria per cubic centimeter during the summer of 1906. Commenting upon these figures he says:

"So far as numbers are concerned they need not greatly alarm us, for we know that disease is due to agencies and conditions other than merely the presence of enormous numbers of bacteria. By universal consent, however, milk containing excessive numbers of bacteria is unsuitable for infant feeding. . . . As we grow older it seems that the gastro-intestinal mucous membrane becomes comparatively immune or resistant to bacterial action. . . . The number of bacteria in milk is not so important from a public health standpoint as the kind and nature of the bacterial products. But with cleanliness and the liberal use of ice, the total number of bacteria can be kept down, and this affords a mode of protection against the dangerous species and their toxic products. Milk containing few bacteria will contain proportionately few or no harmful varieties."

Best Method for Estimating Bacteria in Milk.—The most approved method of estimating bacteria in milk consists in inoculating a suitable

culture medium with a definite amount of diluted milk, pouring plates and counting the colonies which have developed after a certain period of time. While very superior to making stains from the sediment of 10 c.c. of centrifuged milk, this method, however, does not furnish absolutely true values. In the first place only live bacteria yield colonies, but as they naturally are the chief concern in the count, the dead bacteria are of no great significance. Conditions, however, in which all live bacteria present will develop on the plates can never be created, as some microörganisms are aërobic, others anaërobic, and as different species vary in their optimum temperatures of growth, in their requirements as to the exact reaction of the culture medium, etc. Even counts made of various specimens of milk under absolutely identical conditions may furnish quite diverse results; for instance, one sample may contain a large number of anaërobic bacteria and another very few. Since the plates, however, are prepared for aërobic growth, nothing concerning the number of anaërobes is learned. Furthermore, bacteria generally adhere to each other in little groups which cannot be entirely separated into individual microbes even by energetic shaking. Notwithstanding all these defects the method of preparing plates and counting the colonies which have developed after a certain period gives a relatively accurate estimate of the bacterial content of the milk at the time when the specimen was used in the preparation of the plates.

Steps in the Quantitative Bacterial Analysis of Milk.—1. Collect a sample of milk under all possible aseptic precautions from the specimen to be examined. Samples obtained from so-called loose milk in a big can must generally be removed by the ordinary dipper, but should be at once poured into a sterile bottle, the cork stopper or cotton plug of which is to be removed only long enough to permit the introduction of the milk and then to be at once replaced. Samples taken from bottles or smaller containers should be procured with sterile pipettes, into which the milk is drawn up by suction either with a rubber bulb or by the mouth. If the latter method is used the upper end of the pipette must be closed with a cotton plug, so that no trace of saliva can run into the pipette. In general, however, this method, even when the pipette is protected, is not to be recommended. In the examination of certified milk it is best to take one of the original small bottles without opening it. In whatever manner the sample is procured it must immediately be packed in ice to avoid the danger of a great multiplication of bacteria during the period of time elapsing between the collection of the specimen and its inoculation into culture media in the laboratory. The can or bottle from which the specimen is taken should first be well shaken and agitated. It is also well to take the temperature of the milk finally, because a large number of bacteria in cold milk would generally indicate improper collection, while a large number in warmer milk may simply point to a multiplication of lactic-acid bacteria in milk collected cleanly. After

thoroughly shaking the original bottle the sample from certified milk can best be procured at the laboratory by perforating the cardboard cover with a knife sterilized over a flame of a Bunsen burner and inserting the sterile pipette through the hole. In this way all danger of increasing the bacterial contents of the milk during manipulation is entirely avoided. Samples should be taken in quantities of not less than 10 c.c. because of the need for duplicates, controls, etc. The sterile pipettes used in the collection of samples outside of the laboratory should be carried in metal boxes or in sterilized glass tubes. To use a single pipette for collecting a number of samples and sterilizing it between samples by dipping into sulphuric acid and then into sterile water is not a good practice.

2. Milk contains too many bacteria to allow it to be mixed in quantities of 1 c.c. with the culture media employed, hence it must be diluted with sterile water. The water can be kept in ordinary glass bottles. If volumetric flasks are used considerable space should be left over the 10 c.c. or 100 c.c. mark, so that the dilute fluid can be well shaken. The dilutions used are 1 in 10,[1] 1 in 100, 1 in 1000, 1 in 10,000, 1 in 100,000, and 1 in 1,000,000. For the examination of market milk the dilutions are generally 1 in 1000, 1 in 10,000, and 1 in 100,000. Sterilize 9 c.c. and 99 c.c. of ordinary clean tap water in bottles closed with cotton plugs. As a rule, if 100 c.c. are placed in bottles and sterilized in the autoclave for a sufficient time the water will be reduced by evaporation to about 99 c.c. Preliminary tests, however, must determine how much water should be taken to be exactly reduced to 9 c.c. after the sterilization. The dilutions are made in the following manner: Add 1 c.c. of the milk which has been well shaken, with a sterile graduated pipette to the bottle containing 9 c.c. and also 1 c.c. to the bottle containing 99 c.c. of sterile water; shake well for several minutes, taking care that the fluid does not come in contact with the cotton plug. This gives the dilutions of 1 in 10 and 1 in 100. From these the dilutions of 1 in 1000 and 1 in 10,000 can be prepared and from these again the dilutions of 1 in 100,000 and 1 in 1,000,000.

3. Before the dilutions are made the culture media must be prepared. The medium generally used is 10 c.c. of an agar medium containing 1 per cent. of agar, with a reaction of 1.5 per cent. acid to phenolphthalein. The medium should be melted in a water bath, then cooled down to 45° C. To control the temperature exactly a thermometer should be placed in one of the agar tubes in the water bath. When it registers 45° it indicates that this is also the temperature of the agar in the other tubes. The tube containing the thermometer is, of course, not used for pouring plates.

4. When the media are melted and of the proper temperature, place with sterile pipettes 1 c.c. of the dilutions (for example, those of

[1] Only applicable in the very best forms of certified milk.

1 in 1000, 1 in 10,000, and 1 in 100,000) into the lower portions of Petri dishes.[1] Then add the melted agar; mix well with the 1 c.c. of diluted milk in the Petri dish by properly moving and shaking. This should be done carefully so that the medium does not run over.

5. As soon as the agar has again become solid the Petri dishes are inverted, the now upper portion with the solid medium in it is lifted away from the lower portion. Into the latter a piece of filter paper with a drop of glycerin on it is placed. This arrangement insures against moisture collecting on the agar and spoiling the count by spreading the colonies in a diffuse manner. The Petri dish is now placed in an inverted position (culture medium above, plate with filter paper below) into the incubator, and is kept there at 37° C. for forty-eight hours. Another method recommended to prevent the collection of moisture on the agar is to use a porous earthenware cover for the Petri dish. The former method, however, is preferable to the use of a non-transparent cover.

6. After forty-eight hours the colonies on and in the depth of the agar are counted in the manner described in Chapter XIV, p. 174. It is well, however, to count all the colonies which have developed and not merely a number in a portion of the agar. A so-called blank control should be made for each set of specimens. This is done in the following manner: One c.c. of the sterile water to which no milk whatever has been added is poured into a Petri dish and then the melted agar is added. The plate is incubated with the others and examined after forty-eight hours. It should be entirely sterile, or it may perhaps have developed one or two colonies on the surface, which might possibly be due to air contamination during manipulation. The count is made on those plates which have developed between 200 and 400 colonies. This is considered to be the dilution which gives the most trustworthy count from which to calculate the bacterial contents of the milk.

Heinemann and Glenn have made some experiments to determine whether it is preferable, in order to get an exact count, to incubate at 20° C. or at 37° C. They found that in dextrose-litmus-agar[2] the number of colonies after one day is larger at 37° C.; after two days the number is higher at 20° C., and after three days the number of colonies at 20° C. is about double that at 37° C. In lactose agar the conditions are practically the same. There are no acid colonies in either dextrose or lactose agar after twenty-four hours at 20° C. After two days the number of acid colonies in both dextrose and lactose agar is considerably larger at 37° C. than at 20° C., but after three days the proportion is reversed, as the acid colonies develop more rapidly after two days at 20° than at 37° C. The proportionate rate of

[1] Two Petri dishes should be prepared from each dilution.

[2] The culture media used in these tests were prepared as follows: A sterile litmus solution of Merck's pure extract of litmus was poured into a Petri dish, then the dilute milk was added, and finally the melted dextrose or lactose agar was poured into the dish.

acid colonies, however, compared with the total colonies developed is smaller at 20° C. than at 37° C. Heinemann and Glenn, therefore, favor an incubation at 20° C. for three days over one at 37° C. for two days, and they think that dextrose-litmus-agar is better than lactose-litmus-agar, because more acid producers develop on the former than on the latter. For incubation at 20° C. they used an ice-chest kept cool by circulating tap water and heated by an incandescent electric light connected with a thermoregulator, which disconnected the current whenever the temperature rose above 20° C.

Interpretation of the Results of Bacterial Counts in Milk.—The question of the number of bacteria found in milk is, of course, not alone of theoretical but also of great practical interest. Milk containing a great number of bacteria may be unwholesome, and in that case should be condemned and exluded from use as a food. Several cities in the United States have fixed an arbitrary standard of maximum count beyond which milk shall be condemned. It appears, however, that the consensus of opinion among authorities on the question of milk hygiene is that milk cannot be judged from a mere bacterial count as to its fitness or non-fitness for human consumption. Rosenau's opinion as to the significance of large numbers of bacteria in milk has already been given; others have expressed similar or even more pronounced views.

Rodgers and Ayers, for example, state: "Numerical bacteriological standards which are unquestionably of value are necessarily arbitrary, and are based on the count of total bacteria only. Special methods are necessary to obtain any insight into the relative numbers of bacteria of the different groups occurring in milk, and by the information thus obtained to form an opinion regarding the cleanliness and care observed in producing and marketing the milk. . . . A count of the total bacteria does not always give a true indication of the conditions under which milk is produced. In order to interpret results intelligently it is necessary to know, if possible, the age of the milk and the temperature at which it has been held. Clean milk which has been held several hours in a warm place may contain more bacteria than dirty milk when fresh or even after two or three days if it has been held at a low temperature."

Swithinbank and Newman, after giving figures of bacteria found in milk in various cities, say: "Many similar investigations with very similar results might be quoted, but the above will suffice to convey an impression of the bacterial contents of many milks. It is, of course, needless to add that quantitative records, whether represented by high or low figures, are in no sense an exact index as to the injurious nature or otherwise of the milk in question, or as to its value for human consumption. A knowledge of the exact quality of the milk, of the kind of organisms and their role, is necessary before any valid conclusions can be drawn. . . . The fact is that numerical estimation of organisms is not, by itself, a sufficient criterion.

All the circumstances must be taken into consideration, including the condition of the farms, the presence of preservatives, and the species of the bacteria."

Weigmann, in Sommerfeld's *Manual on Milk*, says: "Reports as to the number of germs in milk naturally are very variable; they simply indicate up to the present time that the bacterial contents are occasionally very high. This in most cases is not of as bad a significance as the figures make it appear. However, such figures generally point to an unclean method of obtaining the milk, and this will betray itself generally by the presence of a larger amount of dirt or they point to a not very careful or not rational method of treating the milk."

Conn's opinion is expressed as follows: "It is probably impossible to fix upon any standard as to the number of bacteria which wholesome milk may contain. Should we condemn milk when it has 10,000 per c.c. or 30,000 or 1,000,000 bacteria? To fix a standard is difficult, because the number is so dependent upon the temperature and the season of the year. . . . Sometimes this number (of bacteria in special milk) has been fixed at 10,000, in other cases at 30,000. This is practicable for small dairies where the dealer wishes to furnish a special product at a special price, and where the dairy is within a short distance of the consumer. . . . But for the general milk supply of a large city it has, up to the present time, been found quite impracticable to suggest any bacteriological standard without excluding too large a portion of the milk which will be brought into the city. Moreover, it seems by no means sure that such a standard would be of much practical value, because even though the number be large the milk may be perfectly wholesome if they are of the normal lactic type; whereas a much smaller number of bacteria in another sample of milk might make it decidedly injurious if the bacteria should be of a different character.

"These various facts raise the question whether a bacteriological analysis which shall differentiate the different kinds of bacteria from each other is possible and practical. Is it possible to devise some means of analysis of the bacteria in milk which shall give the numbers of the different kinds of bacteria, separating the normal forms from those that render the milk suspicious? If we could do this, the practical analysis of city milk might be more useful and might become an efficient means in the hands of boards of health in protecting the public from the dangers in its milk supply. There has hitherto been no attempt made to develop such a method of differential analysis of milk, and, indeed, at the present time we know too little in regard to the relations of the different species of bacteria to the wholesomeness of milk to make an analysis absolutely reliable."

Jensen expresses himself as follows concerning this question: "Of course, the number of bacteria in market milk varies greatly according to its care and to the temperature of the air. Experience

gained in most of the larger cities shows the bacterial content of market milk to be seldom below 50,000 to 100,000 per c.c., but it is often greater, varying between 1,000,000 to 30,000,000; indeed, not infrequently, even from 100,000,000 to 150,000,000 have been found, and such milk may not be noticeably tainted. . . . It is known that sour milk has no harmful effect on healthy people. But it is different with those suffering with catarrh of the stomach, and even with small children. . . . The number of bacteria in milk does not give us a safe criterion in this connection, but the degree of acidity furnishes a reliable guide."

Sommerfeld's *Manual on Milk* contains the laws governing the milk supply of the German Empire and of a number of the states forming it, as well as the ordinances, rules, and regulations of two hundred cities. Yet not a word is found anywhere in them about a limit of a permissible bacterial count, while inspections of the cows and dairies by competent veterinarians and detailed rules as to cleanliness, handling, cooling, bottling, labelling, and selling of the milk are amply provided.

It is quite evident that there should be no ironclad rule as to the number of bacteria permissible in ordinary market milk. No competent investigator has ever claimed that a milk containing 1,000,000 bacteria, provided that they are of the ordinary saprophytic kind, normally found in milk, is unwholesome to older children or adults, and it has not even been shown that such a milk is unwholesome to infants. It is, of course, different with the dirty milk containing many millions of bacteria as sold in summer in large cities throughout the world. If it cannot be shown that every milk containing 1,000,000 bacteria is unwholesome to man, why should there be an ordinance condemning such milk? The health authorities of communities should make bacterial counts of milk regularly, because high counts often indicate improper handling, unclean dairies, or cows sick from udder affections. The knowledge gained from bacterial counts should be used to discover such unhygienic conditions, and they should be corrected. But to condemn a milk solely upon the ground that it contains a certain number of bacteria without any further information about it is an inequitable, unjust measure which may lead to the unnecessary destruction of a wholesome food. Metchnikoff and other investigators have for several years advocated the use of milk soured by lactic-acid bacteria as one of the best means to prevent unwholesome fermentations in the human intestines and as one of the most important means to prolong human life. Cultures of lactic-acid bacteria have also been used as sprays in certain pathologic conditions of the nasopharyngeal mucous membranes. So milk containing millions of lactic-acid bacteria and a corresponding amount of lactic acid is not only not unwholesome, but probably (at least, according to Metchnikoff and his followers) a food particularly well adapted to promote longevity. Milk, however, which has already attained a

higher degree of acidity should not be permitted to be sold as fresh, sweet milk, and health authorities should include the determination of acidity among the measures governing the sale of milk.

Determination of the Acidity of Milk.—The acidity of milk can be determined as follows: Place 50 c.c. of milk into a beaker and add 2 c.c. of a 2 per cent. alcoholic solution of phenolphthalein as an indicator. Then add from a burette (gradually under constant shaking or stirring with a glass rod) decinormal solution of sodium hydroxide ($\frac{N}{10}$ sol. NaHO) until all acid has been neutralized and the fluid retains a very faint pink color. This, of course, means that the phenolphthalein in solution now indicates that all acid has been neutralized and that a trace of alkaline decinormal solution of sodium hydroxide has been added in excess. Each cubic centimeter of $\frac{N}{10}$ sol. NaHO is equivalent to 0.009 gram of lactic acid.

In order to obtain the amount of acidity in 50 c.c. of milk each cubic centimeter of decinormal solution used out of the burette to effect complete neutralization must be multiplied by 0.009. The product should again be multiplied by 2 in order to obtain the amount of acidity per 100 c.c. of milk.[1] The rule, therefore, when 50 c.c. of milk have been used is simply: "Multiply the number of cubic centimeters of $\frac{N}{10}$ sol. NaHO used out of the burette by 0.018; the result will be the percentage of acidity in milk." In this calculation the total acidity is expressed as lactic acid; part of the acidity may be due to other fatty acids, such as butyric, succinic, formic, acetic, but this is immaterial, because for practical purposes it is quite sufficient to know the total acidity.

For example, in the test of 50 c.c. of milk, 9.3 c.c. of $\frac{N}{10}$ sol. NaHO out of the burette have been used in order to accomplish complete neutralization; the result then is $9.3 \times 0.018 = 0.167$ per cent. of acid in the milk tested. It is generally held that market milk should not show more than 0.2 per cent. of total acidity, because any excess, as a rule, indicates that lactic-acid fermentation has pretty well begun.

Recording of Results of Bacterial Counts in Round Numbers.—As a rule, bacterial counts of milk are expressed from the number of colonies counted on the plates, in round numbers, so that:

Counts below 100,000 are rounded off in terms of 10,000; for instance, 20,000.

Counts between 100,000 and 500,000 are rounded off in terms of 50,000; for instance, 350,000.

Counts between 500,000 and 1,000,000 in terms of 100,000; for instance, 700,000.

Counts between 1,000,000 and 2,000,000 in terms of 200,000; for instance, 1,400,000.

Counts between 2,000,000 and 5,000,000 in terms of 500,000; for instance, 2,500,000.

[1] It is, of course, customary to express the acidity in per cent., *i. e.*, for 100 c.c. of milk.

Counts above 5,000,000 are expressed in round millions. The following figures, therefore, are used.

Below	10,000	Above	700,000
Above	10,000	"	800,000
"	20,000	"	900,000
"	30,000	"	1,000,000
"	40,000	"	1,200,000
"	50,000	"	1,400,000
"	60,000	"	1,600,000
"	70,000	"	1,800,000
"	80,000	"	2,000,000
"	90,000	"	2,500,000
"	100,000	"	3,000,000
"	150,000	"	3,500,000
"	200,000	"	4,000,000
"	250,000	"	4,500,000
"	300,000	"	5,000,000
"	350,000	"	6,000,000
"	400,000	"	7,000,000
"	450,000	"	8,000,000
"	500,000	"	9,000,000
"	600,000	"	10,000,000

Counts on certified milk should be made and expressed as exactly as possible; so that if a count on a plate from a milk diluted ten times has been made and shows 321 colonies the figure given is 3210 per 1 c.c. of milk.

CERTIFIED MILK.

By certified milk is understood a milk produced under the supervision of a Medical Milk Commission which has established a certain standard to which the milk must conform, and which has issued a set of rules according to which the dairy furnishing the milk must be conducted. These rules generally include the following points: The stables of the dairy must be thoroughly hygienic, the drainage perfect, and the water supply first class. The stock should be subjected at regular intervals to the tuberculin test and be under the almost constant supervision of a competent veterinarian. All sick or suspicious cows should immediately be removed from the herd and new stock, before being allowed to enter, thoroughly examined. The milk should be drawn by perfectly clean milkers who are free from disease themselves. If smallpox, typhoid fever, diphtheria, scarlet fever, measles, and other contagious diseases occur in the vicinity of the dairy a strict supervision should be established and the milkers not allowed to come into contact with persons sick with these diseases, nor may they enter places in which such diseases exist. The hands of the milker and the udder of the cow should be cleansed before milking and the milk received into sterile receptacles, strained through a fine wire gauze, and a layer of absorbent cotton, distributed to sterile bottles and cooled down at once to 50° F. or lower. The bottles must be sealed in a manner preventing subsequent contamination, kept

cool, packed in ice during transportation except in winter, and reach the consumer within thirty hours after being drawn.

Milk commissions generally employ a veterinarian, a bacteriologist, and a chemist as experts for the control of the animals kept in the dairy and of the milk furnished by them. "The duties of the veterinarian," as defined by Ward, one of the foremost American experts on milk, "are to determine the general health of the animals, to observe the sanitary conditions, and to scrutinize the technique of milk handling. In general, his duty is to determine if the conditions of the agreement of the dairyman with the commission are being observed. His criticism and suggestions must maintain that degree of alertness on the part of the foreman of milkers and other employees that shall minimize the possibility of contamination of the milk. The control of bovine tuberculosis is a task that demands the utmost vigilance. Without care in regard to this disease the pretentions of a certified dairy are fraudulent. When not vigorously dealt with it constitutes the greatest menace to the financial success of a certified dairy. Tuberculin tests a year apart, with careless supervision of additions to the herd, are useless in a herd that was badly infected at the beginning, for tuberculosis will keep pace with lax efforts directed against it. It is not sufficient to test merely the cows that happen to be in milk at the time of the test. Every dry cow should be included. In an infected herd a test once in six months is regarded as necessary, followed each time by thorough disinfection of the stable. The control of tuberculosis cannot be accomplished by one test, carried out in a perfunctory manner, but the struggle must extend over years. Additions to the herd must be tested with tuberculin, but there is always danger that an animal though not reacting may introduce the disease. On this account it is far better to subject each animal added to the herd to a three months' quarantine with a tuberculin test at the beginning and end of this period. During the period the milk may be used."

Milk sold as certified under the supervision of a medical milk commission should be examined about once a week by a competent bacteriologist and its bacterial content should not be above 10,000. This expensive milk is almost exclusively used for the feeding of infants, and those paying a high price should have full assurance that they get as excellent an article of food as they have a right to expect. In the State of New Jersey, where the movement creating medical milk commissions originated under the leadership of Henry L. Coit, of Newark, special laws have been enacted to protect the sale of certified milk against any product not coming up to the proper standard.

THE PASTEURIZATION OF MILK.

As previously explained, sterilization consists in exposing an object to such (generally thermal) influences that all life in it is destroyed

and that no fermentative, putrefactive, or similar processes can occur in it. It has been shown how bacterial culture media are sterilized so that they may be used for the development of pure cultures. Organic material in general and certain foodstuffs, particularly meat and milk, are excellent soils for the development of a host of microörganisms. Their growth may so change foodstuffs that they become unfit for food both on account of features repulsive to our senses and because they may actually contain dangerous poisons. It has been long known that low temperatures largely prevent putrefactive processes, and it had also been observed that high temperatures may be used for the same purpose. The Japanese have, for a long time been in the habit of heating their rice wine or sake in spring to preserve it during the summer. When Pasteur studied the changes in wine and beer, known as the diseases of wine and beer, due to certain microörganisms which develop subsequent to the alcoholic fermentation of the yeast cells, or saccharomyces, he tried to devise a means of checking such undesirable growth. As he had finally successfully shattered the old ideas of spontaneous generation, and demonstrated the requirements of reliable, absolute sterilization, the latter procedure at once suggested itself. It was, however, soon found that sterilization could not be employed to protect wine or beer against undesirable microbic multiplication and changes, because it destroyed certain valuable properties in these beverages and was too expensive on account of the excessive breakage of closed filled bottles exposed for a considerable time to the action of the temperature of boiling water or steam. Pasteur then devised methods of using temperatures considerably below the boiling point for certain periods of time, which while not producing absolute sterilization, killed most microorganisms and produced conditions under which articles of food acquired more stable keeping qualities. This process is now generally known as *pasteurization*. After medical bacteriology had become firmly established by the work of Robert Koch the dangers which might lurk in infected milk were not only clearly recognized, but were for a time much overestimated, and an agitation for the general sterilization of milk resulted. At one period a great quantity of the cow's milk fed particularly to infants and children, but also to adults, was sterilized by being boiled, often for a considerable time. While this procedure yielded a milk of very excellent keeping qualities, it was soon found to have its disadvantages in that it developed certain features disagreeable to the taste, which after a time made it decidedly distasteful and even repulsive to some people; but still more important were the facts that it became less easily digestible and assimilable, and that it, when fed exclusively to infants and very young children, produced rickets and scurvy, with anemia and other metabolic and developmental disturbances. The use of fully sterilized milk has today been almost entirely abandoned in the feeding of infants, children, convalescents, or invalids, and instead pasteurized milk is advocated by many.

The terms sterilization and pasteurization with reference to milk are today, unfortunately, still used somewhat indiscriminately, and, in fact, no strictly scientific definition of the term pasteurization is in existence. Tjaden, in the chapter on "Sterilization and Pasteurization" in Sommerfeld's *Manual on Milk*, define *pasteurization* as the heating of milk up to 98° to 99° C.; *sterilization* as the heating to the actual boiling temperature or beyond it; and *Foersterization*, as the heating at 60° C. for one hour. Rosenau defines pasteurization as applied to milk in heating it to 60° C. for twenty minutes, followed by rapid cooling.

As the object of pasteurization, Tjaden designates:

1. A better and more complete separation of the milk into its component constituents.
2. To make milk products more tasty and to impart to them better keeping qualities.
3. To improve the keeping quality of the milk as a whole.
4. To destroy disease germs which may possibly be present in milk.

It has been ascertained that the yield in butter fat in milk treated in the centrifuge is much better at 45° to 80° C. than at lower temperatures. Milk products derived from heated milk and treated with pure cultures of certain fermentative bacteria, permitted to act upon cream and casein in the manufacture of butter and cheese, were also found to have a much finer taste and flavor than the same edibles prepared from raw milk in which other bacteria present modify the special fermentations. While pasteurization improves the keeping qualities of milk this is only true as long as the milk after heating is cooled rapidly, kept cool, and not dispensed in dirty vessels which would again lead to contamination and the rapid multiplication of bacteria. Pasteurized milk has lost the power to inhibit the growth of bacteria for some time, and those bacteria which do multiply in it are not the harmless lactic-acid bacteria but the spore-forming, peptonizing, putrefactive bacteria.

The pasteurizers used in the heating of the milk on a large commercial scale are generally of either one of two types. The milk which goes rapidly through an apparatus in a *continuous stream*, is either heated for a very short time to a comparatively high temperature or it remains in the apparatus for a comparatively long time, is agitated during this period, and is kept at a proportionately lower temperature. This is called the *discontinuous method*.

The object is always the destruction of most bacteria of any kind, and the destruction of all pathogenic bacteria. The most important of the latter is the tubercle bacillus. Numerous scientific investigations have dealt with temperatures and periods necessary to destroy it in milk. The results have furnished by no means uniform data, but it appears to be generally conceded (Tjaden, Weigmann, and others) that in the continuous method heating to 85° C. (185° F.) for one to two minutes is generally sufficient to kill tubercle bacilli. Tjaden,

Koske, and Hertel, however, have made a series of experiments with milk from cows with udder and other forms of tuberculosis and have subjected the milk to temperatures of 85°, 90°, 95°, and 100° C. by the continuous and discontinuous method in apparatuses of various construction. Some of the tests furnished the very remarkable result that at any of the above temperatures tubercle bacilli in the milk from cows with udder tuberculosis were not destroyed and were able to produce tuberculosis by subsequent inoculations into guinea-pigs and in feeding to young pigs. Such positive results were obtained in four series of experiments in which the continuous method and 100° C. were used, and in several experiments in which the discontinuous method, temperatures of 98° C., and time exposures from sixty to one hundred and five seconds were employed. The milk samples used in these tests were very bad and some of the resisting tubercle bacilli were evidently inclosed in casein or pus coagula. Yet it cannot be denied that under certain conditions tubercle bacilli in milk will survive exposures to 100° C. and to 98° C. for over one hundred seconds. Foster and DeMan, using milk from tubercular udders, found that tubercle bacilli were killed at the following temperatures and periods:

At 55° C. (131° F.) after four hours.
At 60° C. (140° F.) after one hour.
At 65° C. (149° F.) after fifteen minutes.
At 70° C. (158° F.) after ten minutes.
At 80° C. (176° F.) after five minutes.
At 90° C. (194° F.) after two minutes.
At 95° C. (203° F.) after one minute.

That commercial pasteurization, as frequently practised in this country, does not kill all tubercle bacilli in milk has been proved a number of times by guinea-pig inoculation of pasteurized milk.

The heating of the milk in pasteurization produces certain changes. When milk is heated by the continuous method to 85° C., but cooled rapidly, little change in taste and smell is produced, though a trace of "boiled taste" may be noticeable. This is, as a rule, more marked in the longer heating at lower temperatures. The same is true of cream and skimmed milk after pasteurization. The formation of cream occurs somewhat more slowly in heated than in non-heated milk, but the yield in butter fat is somewhat larger in the former, as already stated. The greatest effect of the heating of the milk in pasteurization is undoubtedly exerted upon the enzymes of the milk. Even if they are not all completely destroyed their action is undoubtedly much weakened and modified so that from a purely physiological standpoint pasteurized milk is of inferior value in the nutrition of infants when compared with first-class raw milk. Tests have been devised particularly for one of the groups of enzymes in milk, *i. e.*, the peroxydases which are oxidizing ferments and transfer the oxygen in metabolic processes of the organism. These tests can be used to discover whether milk has been heated to a certain temperature.

Storch's Test for Enzymes in Milk.—This test is generally employed in testing milk for the presence of the peroxydases. It is made as follows: Take 10 c.c. of milk in a test-tube, add one or two drops of dilute hydrogen peroxide (H_2O_2), and mix well by shaking, then add two drops of a 2 per cent. solution of paraphenylendiamine and shake again. In raw milk an indigo-blue color is produced, depending upon the presence of active oxydases. The test may also be made, according to Rullman, as a contact ring test as follows: Take 10 c.c. of milk in a test tube, add 10 drops of a 3 per cent. solution of hydrogen peroxide, and shake well. Hold test-tube very obliquely and add slowly from a pipette 1 c.c. of a 2 per cent. solution of paraphenylendiamine, allowing it to flow along the glass on the surface of the milk. A grayish-blue ring is formed at the zone of contact between the milk and the test fluid. According to Kastle, the reaction is delayed in milk which has been heated to 70° C. for fifteen minutes, while it is absent after heating to 70° C. for one hour. It is somewhat delayed after heating to 60° C. for one hour, but not affected after thirty minutes' heating to 60° C.

Home Pasteurization.—Directions for the home pasteurization of milk as given by Rogers in Circular No 152, of the Bureau of Animal Industry, are as follows:

"Milk is most conveniently pasteurized in the bottles in which it is delivered. To do this use a small pail with a perforated false bottom. An inverted pie tin with a few holes punched in it will answer the purpose. This will raise the bottles from the bottom of the pail, thus allowing a free circulation of water and preventing bumping of the bottles. Punch a hole through the cap of one of the bottles and insert a thermometer. The ordinary floating type of thermometer is likely to be inaccurate and if possible a good thermometer with the scale etched on the glass should be used. Set the bottles of milk in the pail and fill the pail with water nearly to the level of the milk. Put the pail on the stove or over a gas flame and heat it until the thermometer in the milk shows not less than 150° F., nor more than 155° F. The bottles should then be removed from the water and allowed to stand from twenty to thirty minutes. The temperature will fall slowly, but may be held more uniformly by covering the bottles with a towel. The punctured cap should be replaced by a new one, or the bottle should be covered with an inverted cup.

"After the milk has been held as directed it should be cooled as quickly and as much as possible by setting in water. To avoid danger of breaking the bottle by too sudden change of temperature this water should be warm at first. Replace the warm water slowly with cold water. After cooling, milk should in all cases be held at the lowest available temperature.

"This method may be employed to retard the souring of milk or cream for ordinary use. It should be remembered, however, that pasteurization does not destroy all bacteria in milk, and after pasteur-

ization it should be kept cold and used as soon as possible. Cream does not rise as rapidly or separate as completely in pasteurized milk as in raw milk."

Advantages and Disadvantages of Pasteurization.—Pasteurization has its advantages and its disadvantages. It has its enthusiastic advocates and those who are opposed to the wholesale pasteurization of the milk supply of a big city. Rosenau gives his views as follows:

"One of the chief objections to pasteurization is that it promotes carelessness and discourages the efforts to produce clean milk. It is believed that the general adoption of pasteurization will set back improvements at the source of supply and encourage dirty habits. It will cause the farmers and those who handle the milk to believe that it is unnecessary to be quite so particular, as the dirt that gets into the milk is going to be cooked and made harmless. It is not proposed that pasteurization shall take the place of inspection and improvements in dairy methods. To insure the public a pure and safe milk supply should be regarded as one of the most important duties of the health officer. Whether pasteurization is adopted by a city for its general milk supply or not, no milk should be accepted that does not comply with certain reasonable chemical and bacteriologic standards. This would aid the inspectors in enforcing good dairy methods. Pasteurization then must not be used as an excuse to bolster up milk unfit for home consumption. To insure this end the health officer should have authority to condemn and destroy bad milk, whether or not pasteurization is practised.

"There is a prevalent impression that the pasteurization of milk improves that important article of diet. Heating does not render milk better in any way as a food. All it does is to destroy certain bacteria and some of their toxic products. It checks certain processes of fermentation and putrefaction, thus rendering the milk safer. On the other hand the evidence seems clear that the pasteurization of milk at 60° C. for twenty minutes does not appreciably deteriorate its quality or lessen its food value.

"Theoretically, pasteurization should not be necessary; practically, we find it forced upon us. The heating of milk has certain disadvantages which must be given consideration, but it effectually prevents much disease and death, especially in infants during the summer months."

Among the objections to pasteurization, Jensen mentioned the following: "Even by the use of a self-regulating pasteurizer it is difficult to provide absolute guarantee that all milk has been heated to the required temperature. To a certain degree pasteurization may conceal a tainted condition, which exists before heating. Quite an abundance of bacteria of putrefaction and other bacteria may be present or the lactic-acid fermentation may have begun to take place; these bacteria are killed by pasteurization; consequently the fermentation and changes that were under way are interrupted.

Under such circumstances one cannot tell by the appearance or taste of the milk that it is damaged and that it contains the product of decompositon of the albumin, or possibly even toxic substances. On the whole there is no way, at the present time, of determining whether or not pasteurized milk was damaged before it was heated, while with respect to raw milk the keeping quality and bacterial content furnish sufficient evidence regarding its true condition. The bacteria surviving pasteurization are, for the most part, the quick-growing bacteria of putrefaction which are inhibited in raw milk by the lactic-acid bacteria, but in pasteurized milk they multiply very fast and undoubtedly they are capable of generating poisonous substances. It has been suggested, therefore, that a pure culture of lactic-acid bacteria be added to milk after pasteurization in order to check the bacteria of putrefaction. In purchasing pasteurized milk one cannot tell if it be fresh or old and cannot determine from its appearance whether putrefaction has begun or if only a few bacteria are present. If we compare the advantages and disadvantages it will be found that there is serious doubt as to whether it is advisable to endeavor to obtain general pasteurizaton of market milk, as has been suggested in Germany."

While there can be no objection to the home pasteurization of milk, in order to destroy pathogenic bacteria which might be present, and to impart to milk better keeping qualities, compulsory pasteurization of most of the market milk supply, decreed by city ordinances has appeared very objectionable to the author, and he has had occasion to express his views on such measures as they have been enacted in the city of Chicago.

Summary of Objections.—A summary of these objections against the wholesale pasteurization of the milk supply of a big city is contained in the following paragraphs:

1. It is known that the exclusive feeding of sterilized or pasteurized milk to infants and children has a tendency to produce rickets and scurvy. This is due to the fact that any effort at pasteurization which will destroy a high percentage of bacteria will also destroy several very important soluble ferments or enzymes contained in milk. The latter are absolutely necessary for the proper nutrition of the infant body, which does not yet furnish these ferments, as is done in later life. The production of rickets has been frequently observed not only in man but as well in some of the lower animals, particularly in small, fancy, high-bred dogs, which often have to be brought up on pasteurized milk because the mother's milk is secreted in insufficient quantity and the puppies do not well tolerate raw cow's milk. On the other hand it is claimed that the feeding of pasteurized cow's milk to calves has not been followed by any evil consequences to the cattle stock of Denmark, where this method of feeding has been practised quite extensively for a number of years. However, observations made on calves fed with pasteurized cow's

milk cannot be made applicable to human infants fed on the same article of diet.

2. Pasteurization of milk makes a subsequent bacteriological examination and estimation of milk, as it originally was, impossible. The evidence of the already undesirable and spoiled character of the milk will be destroyed by pasteurization.

3. Pasteurization of milk practically destroys the so-called lactic-acid bacteria; hence, pasteurized milk will not easily turn sour, but it will undergo putrefactive changes, which, while not readily apparent to the senses of taste and smell, make it a very improper and dangerous article of diet for the feeding of infants and children.

4. It appears impossible to control, at all times, the whole supply of pasteurized milk of a large city; hence, there is great danger that much improperly pasteurized milk may be passed through its market.

5. Pasteurization does not attack the evil of milk from tubercular and otherwise diseased cows at its root, but can at best be looked upon as a makeshift to lessen the dangers of a milk which should from the beginning have been condemned as an improper food for infants and young children.

6. Pasteurization is chiefly directed against the dangers of spreading tuberculosis from the cow to the infant and the child. It has been frequently shown that ordinary commercial pasteurization, as practised in some of the cities of this country, does not safely kill the tuberculosis germ, and milk which has been sold as pasteurized, for instance, in the city of New York, has effectively infected experimental animals with tuberculosis.

7. It has been noted in Rochester, N. Y., and in other cities, that compulsory pasteurization has caused great deterioration in the character of the milk supply furnished to the markets where such laws were in force.

8. The only proper measure to improve the character of the milk supply and to safeguard the people against the dangers which lurk in tuberculosis and otherwise infected milk is to tuberculin-test milch cows; to inspect dairies thoroughly, and to enforce rules, requiring dairymen to keep only healthy cows under proper hygienic conditions and under constant veterinary supervision.

QUESTIONS

1. Do the milk-ducts of the udders of healthy cows contain any bacteria? Discuss the bacterial content of the fore-milk.
2. What is the first effect of raw milk upon the bacterial content? What is this effect due to?
3. What is the effect upon the bacterial count of keeping milk under lower and higher temperatures?
4. Discuss the significance of the number of bacteria in milk according to the views of various authors.
5. Describe in detail the method of estimating the number of bacteria per 1 c.c. of milk.

6. Does this method furnish absolutely accurate values? If not, why not?
7. Describe in detail the method of estimating the acidity of cow's milk.
8. How are the results of bacterial counts rounded off when recording the results of bacterial milk examinations?
9. How are bacterial counts of certified milk expressed?
10. What is certified milk?
11. Give in outline the rules under which certified milk should be produced.
12. What are the duties of the veterinarian in connection with the production of certified milk?
13. Where does the term pasteurization come from?
14. Give a definition of it.
15. What is the difference between sterilization and pasteurization?
16. What is the difference between continuous and discontinuous pasteurization?
17. What are the four objects of the pasteurization of milk?
18. What exposures to heat are necessary to kill tubercle bacilli in milk?
19. Under what conditions are tubercle bacilli in milk particularly difficult to destroy?
20. Has commercial pasteurization in the past been generally a guarantee for the destruction of the tubercle bacilli in milk?
21. What is the effect of a reliable pasteurization upon the enzymes in milk?
22. What are enzymes?
23. Describe Storch's test for detecting peroxydases in milk?
24. Describe Rullman's modification of the Storch test.
25. What is the effect upon peroxydases in milk if the latter is heated for one hour at 70° C.?
26. Describe the method of home pasteurization of milk.
27. Discuss the advantages and disadvantages of pasteurization.

CHAPTER XLVIII.

BACTERIA IN BUTTER AND CHEESE-MAKING.

THE most important products prepared from milk, which is not consumed as such, are butter and cheese. The former may be obtained from sweet milk, sour milk, sweet cream, or sour cream. Cream may be permitted to separate spontaneously from milk, but this is rarely done today, when most cream is obtained by centrifuging the milk in a centrifuging apparatus or cream separator. This has the advantage that the separation occurs more rapidly and is more complete; in fact, apparatuses have been constructed which will leave in the skimmed milk only 0.06 to 0.12 per cent. butter fat and will remove from the whole milk with the cream over 96 per cent. of the butter fat. Butter is usually prepared from sour cream. The souring of the cream may have been permitted to occur spontaneously, generally within thirty to thirty-six hours, by the action of lactic acid bacteria, or it may have been brought about by the addition of a so-called "starter," or "Säurewecker" (German). This starter consists of whole milk or skimmed milk which has become sour spontaneously or a culture of lactic-acid bacteria. The employment of the artificially prepared starters owes its origin to the investigations of Storch, Weigmann, and Conn. Storch, in Denmark, and Weigmann, in Germany, have shown that the souring of the cream was due to lactic-acid bacteria, and Leichmann had demonstrated that it was particularly due to the *Streptococcus lacticus*. Cultures of this organism, also containing some other bacteria and yeast cells which have the power to produce an agreeable aroma in the butter, have been used more and more during the last twenty years in souring the cream for the preparation of butter. It was found, however, that the aroma microörganisms must be used very carefully, since some of them, particularly under certain conditions, have a tendency to split some of the butter fat, forming butyric acid and making the butter rancid. It has also become a practice frequently to pasteurize the milk or cream which is subsequently soured by the starter. Aside from hygienic considerations the advantage of this is that the souring is produced by definite organisms and not by a variety contained originally in the milk, which might impart undesirable flavors or other objectionable properties to the butter. The sour cream is subsequently churned in apparatuses of various types, in which it is subjected to violent agitation and in consequence the globules of butter fat become confluent and most of the watery part of the cream

becomes separated as buttermilk. The latter generally has the following composition: Water, over 90 per cent.; fat, about ½ per cent.; nitrogenous compounds, 3.4 per cent.; lactose, 4.7 per cent.; ash, 0.7 per cent.

The artificial starters used in the souring of cream as employed in this country are either in the form of a powder or in the form of a liquid culture. They are first increased before being added to the cream, a procedure called the "building up of the starter." This is done by adding a freshly opened package or bottle of the starter to a quart of skim milk, whole milk, or cream which has been sterilized or pasteurized and cooled down to 60° F., and stirring and mixing it thoroughly with the milk. The latter is then kept at 65° F., protected against dirt and other contamination. When quite sour, but before coagulation has occurred, the increased or built-up starter is added to the cream which is to be soured or ripened. If the cream used has been pasteurized or has come from pasteurized milk, more of the starter is needed than if this is not the case, and thus, according to varying circumstances from 4 to 10 per cent. of the starter are added. The use of pasteurized cream for butter making is very prevalent in some European countries, because the butter so obtained, owing to the non-development of certain undesirable bacteria, is more uniform in character and less liable to show objectionable features. In the United States artificially prepared starters are often used on unpasteurized cream. In such cases the bacteria of the starter act in combination with the bacteria already present in the cream and the results are not as satisfactory as when pasteurized cream is used.

Conn (*Agricultural Bacteriology*), in discussing the use of starters in our country, says: "The fact that starters, with or without pasteurization have become almost universally used among the better class of creameries is in itself sufficient proof that they are of practical value. Their advantage lies in four directions:

"1. They enable the buttermaker to handle his cream more easily and uniformly. He can regulate the ripening in such a way that his cream will always be of a certain grade of ripeness at a certain time of the day; for a little experience tells him how much of his culture, under proper conditions, should be added to the cream to produce the proper grade of ripening at the particular time when he desires to churn.

"2 The use of starters has produced a greater uniformity in the grade of butter. The buttermaker can depend more certainly upon producing butter of a high grade, month after month, than he can without the starter. There is a general belief also among those who have tested the butter in countries where starters are widely used that there is an improvement in the average quality of the butter as well as in its uniformity.

"3. It has become pretty definitely agreed that the flavor of butter is improved by the use of such cultures. It is somewhat difficult

to obtain definite proof of this, owing to the uncertainty of scores in butter tests. But the fact that all good dairies use them is sufficient testimony to their value in improving the general quality of the butter.

"4. They are the best means of remedying butter faults. Every creamery has experiences of deterioration in the flavor of the butter without any visible cause. Such troubles are known to be due commonly to the growth of unusual and undesirable bacteria in the cream. When they are discovered the sterilizing of the dairy utensils and the use of a larger quantity of vigorous starter will generally remedy the trouble at once. Moreover, the constant use of a starter goes a long way toward preventing these "faults." It is doubtful whether the use of starters produces a butter of a character superior to the best butter made without them. Indeed, some think that it is not quite equal to the best butter made without starters. But the uniformly high grade of culture butter is admitted, and the greater satisfaction in being able to control the process has caused the wide adoption of starters among buttermakers."

Bacteria in Cream and Butter.—The ripening of the cream is due to bacterial growth, chiefly lactic-acid bacteria, with the formation of lactic acid from lactose. The proper ripening of the cream, however, cannot be brought about by simply adding to it a certain amount of lactic acid. This shows that a variety of bacterial enzymes are active in ripening cream so that it will be in the best shape for butter-making; this is generally the case when the acidity present is equal to 0.5 to 0.65 per cent. An enormous increase of bacteria occurs in cream during the changes which lead to ripening. From 2,000,000 to 3,000,000 bacteria per c.c. may have been present in the sweet cream, and when it is ready or ripe for butter-making the number may have increased to several hundred million per c.c. and even to 2,000,000,000. The ripening is best allowed to continue at 65° F., because at that temperature the danger of development of undesirable butter-spoiling bacteria is much less than at higher temperatures. The growth of bacteria in ripening cream is generally stopped by churning; many of them are removed with the buttermilk and more with the subsequent washing and kneading of the butter. While butter is being kept the number of bacteria rapidly decreases, particularly in salted butter. Conn gives the following examples: Number of bacteria present per gram of butter two hours old, 54,000,000; one day old, 26,000,000; four days old, 2,000,000; thirty days old, 300,000. There are, however, some bacteria present even in very old butter. In order to protect butter against subsequent changes it must be kept at a low temperature and protected against light and air. If this is not done it will soon become rancid, *i. e.*, some of the butter fat is decomposed and changed into butyric acid, a fermentative process due to certain bacteria and their special enzyme.

Bacteria and Other Microörganisms in the Ripening of Cheese.—*Proteids in Milk.*—Cow's milk contains about 3 to 4 per cent. of nitrogenous organic compounds or proteids, and these are, as shown by Hammarsten, not of one kind, but three chemically different bodies known as *casein*, *lactalbumin*, and *globulin*. The casein is equal to about 80 per cent. of the entire amount of proteids, and it is present in milk as a calcium compound. It is not in true solution, but in a swollen, finely divided condition known as the colloidal state. When milk is acidulated beyond a certain degree, either by the addition of acid from without or by the growth and development of lactic-acid bacteria, the casein is precipitated as a more or less finely flocculent mass. It is more finely flocculent in human milk, more coarsely flocculent in cow's milk. When this change has occurred the milk is said to have coagulated.

Coagulation of Milk.—Coagulation of milk can be brought about by another procedure aside from acidulation, namely, by the addition of an enzyme called *labferment*, or *rennet*. This enzyme is furnished by the mucous membrane of the stomach of animals, and also by a number of bacteria, as first shown by Ducleaux, who demonstrated that certain bacteria growing in milk coagulate it by the aid of this ferment. Conn succeeded in separating the rennet enzyme from the bacteria which had produced it. While very similar in action the bacterial rennet is not absolutely identical with the rennet obtained from calves' stomachs or the stomachs of other mammals. The bacterial rennet can also coagulate sterilized milk, the latter only raw milk. There are quite a number of bacteria which can coagulate milk by the aid of their rennet enzyme even without acidulating it. Among such bacteria are particularly the potato bacilli (Bacillus mesentericus vulgatus and other bacteria of this group). The rennet prepared from calves' stomachs cannot coagulate boiled milk because it acts only in the presence of soluble lime salts, and these are precipitated when milk is boiled; it can likewise not act in an alkaline solution, but acts best in a slightly acid medium at a temperature of 37° C. At 25° C. the action is slow; at 45° C. it does not take place, and at 70° C. the enzyme is destroyed.

The Formation of the Curd.—In order to prepare cheese, milk may be coagulated by the addition of the lab- or rennet enzyme[1] from calves' stomachs, by permitting it to become sour spontaneously, or by adding milk already soured or a starter. According to the method used in coagulation, cheeses are distinguished as *rennet milk cheeses* and *sour milk cheeses*. The soft, spongy mass, full of fluid, which is formed when milk has been coagulated by either one of the two methods, is called a *curd*. Most of the *whey* may be pressed out of the curd or a considerable portion may be left in it. In the former case the comparatively dry raw material will form the *hard*, in the latter

[1] An exceedingly small amount of rennet will coagulate a very large amount of milk.

case the *soft cheese*. The raw material so obtained is still simply curd and becomes cheese only after going through a process of ripening with various changes depending upon the activity of bacteria and other microörganisms. In addition to the proteids, the curd contains a variable amount of fat, depending upon whether it has been obtained from whole or from partially or more completely skimmed milk.

Ripening of Curd into Cheese.—The ripening of the curd into cheese consists in a partial or more or less complete conversion of the insoluble casein into simpler and soluble proteid or albuminoid bodies and in the production of certain bodies which give to the ripe cheese the peculiar taste and characteristic flavor which vary greatly in different varieties. The change of the casein which resembles that brought about by gastric juice is due to enzymes secreted by bacteria and other microörganisms, which multiply enormously during the process of ripening. It is still a much disputed point to what extent certain bacteria are responsible for the ripening of the cheese. It is believed, however, by many that the hay bacillus (Bacillus subtilis), which secretes a strong proteolytic or peptic ferment, plays an important role in the conversion of casein into soluble albuminoids. In some soft cheeses the presence of Bacillus subtilis in enormous numbers has been established. Ducleaux has shown that peptonizing bacteria which he has named Tyrothrix tenuis, distortus and geniculatus, are important factors in the ripening of certain French cheeses, and Adametz proved the presence of bacteria likewise endowed with proteolytic enzymes in soft and hard Swiss cheeses. It has also been shown that lactic-acid bacteria which at the same time possess the power to produce certain changes in casein likewise play an important role in the ripening of cheese. Liquefying cocci have been found in cheese by von Freudenreich. Thoeni and Weigmann and Jensen have shown that their peptonizing enzyme is an important factor in the early changes in curd. Von Freudenreich and Jensen have also found in Swiss cheese anaërobic bacteria which decompose lactate of lime into propionic acid, some acetic acid and carbon dioxide, and which produce holes in the cheese, improving its flavor and taste. Rodella and Weigmann have discovered anaërobic butyric acid bacilli in Swiss cheese and Paraplectrum fœtidum in Limburger cheese. Weigmann gives the following summary of the activity of bacteria and other microörganisms in the ripening of cheese:

"The lactic-acid bacteria, such as Streptococcus Guentheri and others, which remain in the curd with the larger or lesser amount of whey contained therein, multiply and form lactic acid, this prevents the immediate activity of putrefying bacteria originally contained in the milk and present in the curd. The bacilli most susceptible to acid, such as the Bacillus coli, some of the hay bacilli, and some of the anaërobics present, are much reduced in numbers, liquefying cocci and lactic-acid bacteria, which can decompose casein without peptonization, however, multiply considerably. After the activity

has decreased and after some ammonia has been formed, hay bacilli and certain hyphomycetes become more active, they secrete peptonizing enzymes, and the ripening process makes more rapid progress. The lactose fermentation first occurring in cheese is a most important process, which prevents true putrefactive changes and prepares the field for a ripening of the proper kind. Saccharomyces, oidia and hyphomycetes probably play an important role in the ripening of cheese and in giving it the proper flavor. Saccharomyces very likely produce esters during the acid formation; oidia and hyphomycetes neutralize the acids and give the peptonizing bacteria a chance to multiply and to produce their proteolytic enzyme." It has been shown by Conn and others that Camembert cheese owes its ripening and its flavor and taste mainly to the presence of Penicillium candidum and Penicillium glaucum, while Roquefort cheese owes its properties to the presence of a variety of Penicillium glaucum(Penicillium roqueforti or Penicillium aromaticum casei). The bacteria in ripening cheese, according to Conn, for a number of days, sometimes for several weeks, increase in numbers. After this they decrease until when the cheese is fully ripened they are very few compared to their number at certain stages of the ripening. An examination showed in fresh cheese 6,600,000 bacteria per gram; when four days old, 51,000,000 per gram, and when four months old, 1,000,000 per gram. Faults in cheese, such as a gassy condition, swelling, undesirable flavor, have been shown to be due to undesirable bacteria or torulas. To prevent such faults Conn recommends cleanliness, a vigorous lactic-acid starter for the souring of the milk, and a temperature of 60° F., which is not favorable to those organisms, generally responsible for the faults of cheese.

QUESTIONS

1. How can the cream be separated from the remainder of the milk?
2. What causes the ripening of the cream?
3. How much acidity does ripe cream contain?
4. How can the acidity be ascertained?
5. How can the ripening be produced by the addition of lactic acid?
6. What is a natural starter?
7. What is an artificial starter?
8. What microörganism is more particularly the cause of the ripening of the cream?
9. What is meant by aroma microörganisms?
10. Why is their use sometimes dangerous?
11. Discuss the advantages of using pasteurized cream for butter-making.
12. What is the composition of buttermilk?
13. What is meant by the building up of the starter?
14. What are the bacterial contents of sweet cream and of ripe (sour) cream?
15. Discuss the decrease in bacterial content during butter-making and in the butter after its preparation.
16. What is rancid butter; what causes its production?
17. What are the proteids contained in milk?
18. How is coagulation of milk brought about?
19. What is lab-enzyme or rennet?
20. How can it be obtained?

21. What is known about bacterial rennet?
22. Under what conditions does rennet act?
23. What is meant by the curd? What by whey?
24. What are the constituents of curd?
25. What brings about the change from curd to cheese?
26. What are the essential changes in the ripening of cheese?
27. What role do the lactic-acid bacteria play in the ripening of cheese?
28. What is the role of liquefying cocci?
29. What of peptonizing bacteria?
30. What of moulds, saccharomyces, oidia?
31. What is meant by faults of cheese? What causes them?
32. How can they be prevented?

CHAPTER XLIX.

SIMPLE CHEMICAL MANIPULATIONS—NORMAL SOLUTIONS AND INDICATORS REQUIRED IN LABORATORY WORK IN BACTERIOLOGY.

REFERENCE has been made in the preceding pages to certain simple chemical manipulations and tests employed in standardizing the reaction of culture media, estimating the change in their reaction or that in milk in consequence of bacterial growth, and determining the exact amount of acid or alkali formed. These manipulations require a set of simple chemical apparatus and a few chemical reagents, standard solutions, indicators, etc.

Apparatus.—The apparatus required is the following:

1. A moderate-sized balance of medium delicacy, carrying about 50 grams, sensitive to 1 milligram or less, and a moderately good set of weights from 20 grams to 1 milligram.

2. A number of volumetric flasks holding 1000 c.c., 500 c.c., 250 c.c., 100 c.c., and 50 c.c.

3. A number of pipettes holding 10, 5, 2 and 1 c.c.; one 10 c.c. pipette graduated in $\frac{1}{10}$ c.c. and one 1 c.c. pipette graduated in $\frac{1}{100}$ c.c.

4. A number of graduates, beakers, porcelain evaporating dishes, mortars, flasks, funnels, test-tubes, fermentation tubes, glass and rubber tubing, and glass stirring rods, and a Kipp gas generator.

5. Several burettes. These are used for the delivery of an accurately measured quantity of standard or normal solutions. A burette is made from a long, glass tube of even bore throughout, holding 50 or 100 c.c. On the glass tube lines of division are engraved, corresponding generally to $\frac{1}{10}$ c.c. The outlet of the burette is either a rubber tube with clip or burette clamp and a glass tip or a ground-glass stopcock. Burettes are used in an upright position, held in a burette stand, and the fluids from them are generally delivered slowly, drop by drop, by manipulating the clip, the clamp, or the stopcock.

Gravimetric and Volumetric Analysis.—The method of determining quantitatively a chemical substance by obtaining it first in a pure state or in a compound of known composition and then weighing it on a delicate chemical balance is called the *gravimetric method.* This method, for instance, is used in the laboratory in determining exactly the amount of butter fat in milk, but it is not often used in bacteriology. The method of determining quantitatively the amount of an acid alkali or other chemical compound by the aid of volumetric or standard solutions is called *volumetric analysis,* and this is the method generally employed in bacteriological work.

Normal Solutions.—A normal or standard solution (N sol.) may be defined as one which contains one molecular weight of the reagent in grams dissolved in enough distilled water to make exactly 1000 c.c. at a temperature of 16° C. (60° F.). If, for instance, a *normal solution of caustic soda* or *sodium hydrate* (used in standardizing our culture media) is to be prepared the molecular weight of sodium hydrate, which has the chemical formula NaHO, must first be ascertained. The atomic weight of sodium (Na) is 23, that of hydrogen (H) is 1, and that of oxygen (O) is 16. Hence, NaHO has a molecular weight of 40. In preparing a standard or normal solution of NaHO, therefore, 40 grams of the chemically pure sodium hydrate (NaHO chemically pure) are weighed out, placed in a 1000 c.c. volumetric flask and dissolved in several hundred c.c. of distilled water; the solution (which has become warm) is allowed to cool and then enough distilled water is added to make up exactly 1000 c.c. at 16° C. Sodium hydrate is a *hygroscopic substance*, that is, one which will draw water from the atmospheric air, and in order to obtain an accurate normal solution it must be weighed out rapidly. In fact, in exact chemical work it is impossible, in preparing normal solutions, to begin with a normal solution of sodium hydrate, but another normal solution must first be prepared from a substance which is not hygroscopic and which can be weighed out very accurately. Dry, normal sodium carbonate, Na_2CO_3, is generally used for this purpose. Another factor must be considered in the preparation of normal solutions. The molecular weight in grams (in the above case of NaHO, 40 grams) is to be taken only in case of univalent substances, which contain one hydrogen atom in the molecule, while in the case of bivalent substances, which contain two hydrogen atoms, one-half of the molecular weight in grams is taken. If a normal solution of sulphuric acid, H_2SO_4 (the molecular weight of which is 98), is to be prepared, 49 grams are taken and diluted with enough distilled water to make 1000 c.c. at 16° C. The amounts of the reagents to be taken in the preparation of the few normal solutions used in bacteriologic work are as follows:

	Grams per 1000 c.c.
Crystallized oxalic acid	63.00
Sulphuric acid	49.00
Hydrochloric acid	36.37
Nitric acid	63.00
Water-free sodium carbonate	53.00
Sodium hydrate	40.00
Potassium hydrate	56.00

The student, however, must not suppose that all of these normal solutions can be obtained by simply weighing out the amount indicated and dissolving it in sufficient water to make 1000 c.c. Several of the above chemicals, sulphuric acid and sodium and potassium hydrate, for instance, are very hygroscopic; others, like nitric acid and hydrochloric acid, evaporate and change during manipulation; in fact, the

only substance which can immediately be safely used to obtain accurate normal solutions is the perfectly dry, absolutely pure sodium carbonate, Na_2CO_3. Of this, 53 grams are weighed out, placed in a 1000 c.c. volumetric flask, dissolved with several hundred cubic centimeters of distilled water, and made up to 1000 c.c. at 16° C. The next solution to be prepared is one of sulphuric or hydrochloric acid. This must be done in such a manner that 1 c.c. of the alkaline carbonate of sodium solution will completely neutralize 1 c.c. of the acid solution, and vice versa. This, however, is very difficult in practice, and generally there is a slight discrepancy. After much manipulation in the preparation of the acid normal solution it may, for instance, be found that 49.8 c.c. of the latter will just neutralize 50 c.c. of the alkaline normal solution. Such a slight difference can be entirely neglected in bacteriological work; in very exact chemical work, however, correction of the result would be necessary by multiplication of the number of cubic centimeters used out of the burette containing the slightly too strong acid normal solution by $\frac{50}{49.8} = 100.4$. As a rule, normal solutions are not used in full strength, but they are diluted with distilled water in proportions of 1 in 2, 1 in 4, 1 in 5, 1 in 10, 1 in 20, 1 in 100. Such dilutions are called one-quarter normal solution, decinormal solution, centinormal solution, etc., and designated in writing as $\frac{N}{2}$, $\frac{N}{4}$, $\frac{N}{10}$, $\frac{N}{20}$, $\frac{N}{100}$, etc. In bacteriology decinormal solutions ($\frac{N}{10}$ sol.) are generally used and prepared by taking 100 c.c. of the full strength normal solution, pouring it into a 1000 c.c. volumetric flask and making it up with distilled water to 1000 c.c. at 16° C.

When a normal or dilute normal solution is used to find out the exact amount of a certain substance in another solution the latter solution is said to be titrated.[1] In doing so the normal solution is allowed to flow gradually from a burette into an exactly measured amount, say 10, 20, or 50 c.c. of the solution which is being tested. The steps of such a titration have been explained in detail on page 502 in the chapter on Milk in the Quantitative Estimation of Lactic Acid. A titration may also be made by placing a definite amount of the normal solution in a beaker and filling the other solution which contains the substance to be determined quantitatively into a burette and allowing it to discharge gradually into the normal solution until the reaction is complete.

Indicators.—Whenever a titration is made there must be something to indicate when the reaction is complete. If, for instance, a fluid is titrated for the amount of acid which it contains it is necessary to know when enough of the normal alkaline solution has been added to neutralize the acid present. A reagent added to the acid solution

[1] The words titrate and titration are derived from the French word "titre," which means title, power, or strength. Titrating means, therefore, to find out the strength or concentration of a substance in a solution. This word is also used a good deal in serum investigations, as, for instance, to ascertain the titre of an immune serum, etc.

which will not interfere with it in any shape or, form but which will tell when the acid has been completely neutralized, or, rather, when a very small amount of the alkaline solution has been added in excess, is called an indicator. In general, therefore, an indicator is a substance which by a change of color or a precipitate formed or in some other visible manner will indicate the end point of a reaction. It is always best to use indicators with daylight illumination, because artificial light frequently makes the color reaction less characteristic, and, therefore, confusing.

The following are the formulæ for some of the most commonly employed indicators used in the titration of acids and alkalies:

Dimethylamidoazobenzol.—This is a coal-tar derivative (anilin stain) and is used in the proportion of 0.05 gr. in 100 c.c. of 95 per cent. alcohol. It is yellow in neutral and alkaline solutions and red in acid solutions. It is particularly useful in the titration of strong mineral acids, and is generally used in the determination of hydrochloric acid (in gastric juice, natural or artificial, in investigating the effect of gastric juice upon pathogenic bacteria). A few drops of the indicator are added to 10 c.c. of the gastric juice. The fluid in the presence of HCl assumes a red color. $\frac{N}{10}$ sol, NaHO is then added from a burette and each c.c. of the normal solution used in neutralization is equal to 0.00365 gram of HCl.

Cochineal.—This substance is prepared from the cochineal louse (Coccus cacti cochinelifera), living on certain species of cactus. It is the substance from which the carmine used for staining tissues is also derived. Three grams of cochineal are extracted in the cold with 250 c.c. of 25 per cent. alcohol. This cochineal tincture assumes a violet color in the presence of alkalies and a yellow-red color in acid solutions. It is, like diamethylamidoazobenzol, used in the titration of strong mineral acids.

Litmus.—This substance is of vegetable origin and derived from several species of lichens. It is sold in commerce in the form of small cubes or larger cakes. In order to prepare a good indicator used for general laboratory purposes, or for the preparation of litmus-lactose or litmus-glucose agar and gelatin, it is necessary to boil the cubes with three or four changes of 95 per cent. alcohol. This extracts a dirty violet substance from the commercial article. After purification with alcohol the cubes are soaked in water until the latter assumes a dark blue color. The fluid is then drawn off and dilute sulphuric acid is added to it until a deep violet color is produced. In order to secure the proper color it is necessary to take a few c.c., dilute strongly with distilled water, and examine in a test-tube. When the dilute fluid has a reddish-violet tint, almost a cherry red, a sufficient quantity of sulphuric acid has been added to the original watery extract. Litmus tincture or paper is stained blue in alkaline, red in acid solutions. A tincture stained slightly blue in an alkali on standing frequently turns red, and it therefore must be made blue again before

use by the addition of alkali. Litmus is the most commonly used indicator for various acids and alkalies.

Rosolic Acid, or Corallin.—This is an aniline stain. It is dissolved in the proportion of 0.5 gr. to 50 c.c. of 95 per cent. alcohol. After solution 50 c.c. of distilled water is added. The indicator is yellow in neutral and acid solutions and turns rose red in alkaline solutions. It is particularly useful in the titration of organic acids, and is, therefore, used in the determination of lactic, butyric, formic, succinic, and acetic acids.

Phenolphthalein.—This is a coal-tar derivative, and is very much used in work in bacteriology. It is generally employed in the exact titration of culture media, as previously explained in detail. The indicator is prepared as a 1 per cent. alcoholic solution. It is colorless in neutral and acid solutions and turns red in alkaline solutions. The change of color in this solution can also be well seen by artificial illumination. It also takes place promptly in hot fluids, and the phenolphthalein indicator has, therefore, a wide range of application. It can, however, not be depended upon for the determination of ammonia and the weak alkalies, but it is excellent for the hydrates of sodium, potassium, calcium, and barium.

Empirical Standard Solution.—These are based upon a different principle. They are not prepared according to the molecular formula of the chemical compound employed, but in such a manner that 1 c.c. of the standard solution will be equivalent to 10 milligrams of the substance which is to be determined quantitatively by the volumetric analysis.

Fehling's Standard Solution.—A fluid of this type is Fehling's standard solution for the quantitative determination of sugars (glucose, dextrose, maltose, lactose). As the quantitative determination of sugar, either to ascertain how much of it has been fermented by certain bacteria or has been formed from starch, frequently must be made in bacteriological work, the formula for Fehling's copper solution and the steps in the analysis will be given here.

Fehling's Standard Solution:

Solution 1.

Sulphate of copper, chemically pure crystals	34.638 grams
Sulphuric acid, chemically pure	1 c.c.
Dissolved in enough distilled water to make	500 c.c.

Solution 2.

Tartrate of sodium and potassium, chemically pure (Rochelle salt)	175 grams
Sodium hydrate, chemically pure	125 grams
Distilled water enough to make	500 c.c.

Two cubic centimeters of the copper and alkaline solution mixed is equivalent to 10 milligrams of glucose or dextrose, to 16 milligrams of maltose, and to 13.5 milligrams of lactose. Solutions No. 1 and No. 2 must be kept separate. They are mixed only in exactly equal

proportions just before use, because the mixture is not very stable and decomposes. After the two fluids are mixed the copper is present in the form of a hydrate of the metal, and this hydrate in hot alkaline solution is reduced by the sugars named into, first, a cupric oxide, and then a cuprous oxide. The former is a yellow, the latter a red-brown copper salt. The details of this process of reduction are not yet perfectly clear, but it is established beyond doubt that 10 milligrams of glucose, 16 of maltose, or 13.5 of lactose will bring about the complete reduction of all of the copper hydrate contained in 2 c.c. of the mixture of solutions No. 1 and No. 2; that is, of the copper salt originally contained in 1 c.c. of solution No. 1. When the reduction of the copper hydrate occurs in the hot alkaline solution a yellowish or orange precipitate is generally first formed, and finally a red-brown precipitate at the bottom of the beaker; the supernatant fluid after the fluid has become cool is perfectly colorless (all blue color has disappeared). As it would be impossible to recognize at the right moment the end point of the reaction, *i. e.*, the complete reduction of the copper hydrate) an indicator must be used. As such a saturated watery solution of ferrocyanide of potash strongly acidulated with glacial acetic acid is employed in the following manner: Small pieces of filter paper are moistened with the indicator and from time to time a drop of the boiling copper solution (to which the sugar-containing fluid from the burette is being added) is allowed to fall on them. As long as unreduced copper hydrate remains in the boiling solution it forms a red cyanide of copper with the acid ferrocyanide solution. When the reduction, however, is completed no more cyanide of copper is formed and no more sugar containing fluid should be added from the burette.

Steps in the Determination.—1. Mix equal amounts of solutions from bottle No. 1 and No. 2. This mixture is the standard solution now ready for use.

2. Take 20 c.c. of the mixture, place into a beaker, dilute with 30 c.c. of distilled water, and heat over a small flame, or, still better, in a water bath.

3. The sugar containing fluid must first be filtered, and if it contain much sugar (which should be ascertained by a preliminary qualitative test) it should be diluted with distilled water, so that it probably contains between ½ and 1 per cent. sugar approximately.

4. Heat the standard solution in the beaker to boiling and then add the sugar solution gradually from the burette. Continue heating and adding, and from time to time test the boiling solution with the indicator. When cyanide of copper ceases to be formed on the moistened filter paper, stop adding sugar solution from the burette. If the test has been made properly the fluid in the beaker should be colorless after cooling, and if mixed with the indicator should not form a red precipitate. Nor should it look yellow, for this would indicate that an excess of sugar solution had been added.

5. After the completion of the reaction, read off from the burette the number of c.c. of sugar solution used and calculate from this figure the percentage of sugar present in the original sugar-containing fluid.

Example.—As an example, suppose that a 3 per cent. starch bouillon has been inoculated with a microörganism, secreting a diastatic ferment, and that the amount of sugar formed after three days' incubation is to be ascertained. The solution must first be filtered. The preliminary qualitative test, also made with Fehling's solution, shows considerable sugar present. The filtrate is, therefore, diluted in the proportion of 1 to 3 parts of distilled water and the fluid so obtained filled into a clean burette in which it is so regulated that 40 c.c. are present when the addition to the boiling copper solution is begun. When all the copper hydrate has been reduced it is found that 24 c.c. have been used out of the burette. This amount of fluid contains 100 milligrams of sugar, which are necessary to reduce all of the copper hydrate in 20 c.c. of Fehling's solution; hence, 100 c.c. of a fluid like the one used in the burette contain 416 milligrams. Since the fluid in the burette represents one-fourth of the concentration of the original filtered liquid, the latter contains four times as much sugar as the dilute fluid used in the burette, or 1664 milligrams = 1.664 grams of sugar per 100 c.c., or 1.664 per cent. If n is the number of c.c. used out of the burette then the following equation results:

$$\mathrm{n} : 100 = 100 : \mathrm{x}$$
$$\text{and } \mathrm{x} = \frac{10{,}000}{\mathrm{n}}$$

In other words: To find the amount of sugar in milligrams present in 100 c.c. of the fluid used in the burette, divide 10,000 by the number of c.c. used out of the burette. This gives the amount of sugar in milligrams per 100 c.c. for the dilute fluid, and it must be multiplied by the number of times diluted to express the amount of sugar in milligrams present in 100 c.c. of the original fluid. The sugar in this example has been calculated as glucose or dextrose. In the case of maltose the division is made into 16,000 in the same manner and in the case of lactose into 13,500, because these sugars reduce copper hydrate in a different manner. Saccharose does not reduce copper hydrate, and when it is to be determined by the aid of Fehling's solution it must first be changed into invert sugar by boiling with dilute acids or by the action of the enzyme invertin.

QUESTIONS

1. What apparatus is required for the simple chemical manipulations employed in elementary bacteriology?
2. What is a burette, a pipette, a beaker, an evaporating dish?
3. What is meant by the gravimetric, what by the volumetric method of quantitative chemical analysis?
4. What is a normal solution? What a decinormal, centinormal solution?
5. What is the atomic weight of an elementary body? What is the molecular weight of a compound body?
6. What is the atomic weight of N, O, C, and S?
7. What is the molecular weight of caustic soda?
8. What is the procedure in preparing a set of acid and alkaline normal solutions for use in the standardization of the common culture media?
9. How is the acid standardized against the alkaline solution? Is it easy to prepare them so that 100 c.c. of the acid normal solution will exactly neutralize 100 c.c. of the alkaline normal solution?
10. If they are not exactly balanced what can you do to correct the result?
11. What is a titration?
12. What is an indicator?
13. Describe the preparation of the following indicators: Dimethylamidoazobenzol, litmus, rosolic acid, phenolphthalein.
14. Give the formula for Fehling's standard solution.
15. What is an empirical standard solution?
16. Describe the use of Fehling's standard solution in the quantitative estimation of sugar in a fluid.
17. How much of the following sugars does it take to reduce all of the copper hydrate contained in 20 c.c. of Fehling's solution? Glucose, maltose, lactose?
18. Describe the indicator used in connection with Fehling's solution.
19. How does this indicator work?

PART IV.

PATHOGENIC PROTOZOA.

CHAPTER L.

GENERAL CONSIDERATION OF PROTOZOA—CLASSIFICATION—MORPHOLOGY AND REPRODUCTION.

Definition and Morphology.—Protozoa represent the lowest and most simple form of animal life. The organisms, however, vary much in their morphology. While unicellular and occasionally united in colonies of unicellular individuals, they may have a body with a variety of parts, or small organs called *organella,* serving different purposes. In this respect protozoa when compared with the lowest forms of vegetable life (the bacteria) are considerably higher in structural differentiation. In a summary manner protozoa may be defined as unicellular animal organisms. Calkins gives the following more elaborate definition: "A protozoön is a primitive animal organism, usually consisting of a single cell, whose protoplasm becomes distributed among many free living cells. These reproduce their kind by division, by budding, or by spore formation, the race thus formed passing through different form changes, and the protoplasm through various stages of vitality collectively known as the life cycle."

Protozoa, like the lowest forms of vegetable life, are very prevalent in nature, but they do not find the conditions necessary for or favorable to their nutrition and multiplication as easily as the bacteria. The lowest forms of animal life being highly differentiated and representing a great variety of morphologic features, the phylum protozoa has been divided into several subphyla, numerous classes, subclasses, orders, families, and genera.

Protozoa, like bacteria, live either in the outside world or as parasites on or in other organisms. They may exist as harmless commensales or they may be pathogenic parasites.

The number of protozoa known to be pathogenic to man and the higher animals is comparatively small. As these belong to a few families only, medical and veterinary studies of protozoa, aside from a general survey of the phylum protozoa, may be confined to

the morphology and the biologic characters of a very limited number of families.

Shape and Size.—Protozoa, composed as they are of a soft protoplasm, present themselves under various shapes, depending upon differences in environment; they may be more or less spherical, or *homaxonic*, or they may be decidedly elongated in one axis, or *monaxonic*. When the environments become unfavorable and under other conditions, protozoa often become perfectly spherical, and secrete a thick, resistant membrane composed of chitin. This change is known as the *encysted stage*. The organism may die in it or it may relinquish the encysted condition and return to its former shape, divide under the protecting membrane into daughter cells, or sporulate when circumstances become more favorable. Protozoa are evidently more resistant in the encysted stage, and it may in certain respects be likened to the spore formation in bacteria, though spore formation in protozoa is a process quite different and distinct from encysting.

As a class of organisms protozoa vary so much in shape that no common description will fit all of them. They likewise vary greatly in size (from one or a few micra to several millimeters), and it cannot even be said that all protozoa are microörganisms, as some can be easily seen with the naked eye. *Porospora gigantea*, a gregarina found in the intestines of the lobster, is 16 mm. long. The individual organisms of one and the same species will also frequently vary considerably in size, much more than bacteria. This variability often depends upon the amount of available food supply and upon other external conditions.

Structure.—Bacteria do not yet show a differentiation of the cell into a protoplasmic body and a nucleus; all protozoa, however, possess a distinct nucleus or several nuclei. The cell protoplasm or cytoplasm of protozoa is composed of the *spongioplasm*, which generally has a network or honey-combed structure, and is made up of a rather firm, tenacious substance, and the *hyaloplasm*, which is a more fluid substance contained in and filling out the network of the spongioplasm. The outer layer of the protozoan organism generally is composed of a more condensed and tougher protoplasm called the *ectoplasm* in contradistinction to the softer protoplasm within known as the *entoplasm*. The ectoplasm sometimes secretes or forms a membrane, an armor, organs of locomotion, etc. Round or oval spaces called *vacuoles* are frequently seen inside of the protoplasm. They are not empty or air-containing spaces, but are filled with a watery fluid and are either concerned in the digestion and assimilation of food particles or are contractile vacuoles which empty and refill. Protozoa sometimes contain a complicated system of intercommunicating vacuoles through which fluid more or less constantly circulates. In addition to the vacuoles the cytoplasm of protozoa shows *granules* of various size and shape. Thus the small granules of Altmann, supposed to be intimately connected with the ultimate structure and

function of the protoplasm as such are found; also food particles, pigment granules, oil drops, waste material and foreign material, like lime or silica, more or less accidentally taken up into the body of the organism. The granules, which contain stored food material, are often called *plastids*, and plastids containing pigment are known as *chromatophores*.[1]

Nucleus and Nuclear Substances.—Protozoa generally contain two nuclei. In some protozoa these cannot be well recognized as two distinct nuclei, because they are, during the period of rest, contained in one common nuclear membrane; in other protozoan organisms they can always be well distinguished. Since one of the nuclei is generally large and the other small, they have been called the *macronucleus* and the *micronucleus;* the latter is also called the *blepharoblast*, because a flagellum may originate from it. The large nucleus is generally concerned in the nutrition of the organism; hence it is called the *trophonucleus*. The small or micronucleus is clearly concerned in the reproduction and multiplication of the protozoan organism, and, for this reason, Calkins recommends that it be called the *karyogonad*, or the *gonad nucleus*, as representing the germ plasm from which reproduction starts. This gonad nucleus, when contained in a common nuclear membrane with the trophonucleus separates from it during the period of maturation which precedes reproduction. That portion of the nucleus which stains with the so-called nuclear stains (hematoxylin, carmin, the basic anilin stains) is called the *chromatin*. The latter under certain conditions may leave the nucleus and become distributed diffusely in the form of granules in the cytoplasm. Besides the chromatin the nuclei of protozoa contain another very important substance, which apparently is the source of energy of motion and metabolism; this substance has been called *kinoplasm* (also *archoplasm*). Calkins calls this kinoplasm, whether found inside or outside the nucleus, the division centre. Its importance in relation to the function of locomotion is well recognized in trypanosomes where the material of the undulating membrane, the flagellum and the other contractile locomotory structures, is all derived from this division centre, or kinetonucleus.

Organs of Locomotion.—1. *Pseudopodia.*—The most primitive form of locomotion among protozoa is the ameboid motion, depending upon the possession of pseudopodia (false feet). These are simply digit-like prolongations or out-flowings of the cytoplasm. In ameba the latter under favorable conditions is constantly in a flowing motion combined with a constant change of shape. This ameboid motion. by the flowing and drawing action of the contractile protoplasm is not only shown by certain protozoa, but also by some of the cells of higher multicellular (metazoic) animals. The most important cells

[1] The term chromatophore in histology and histopathology of higher animals designates pigment containing cells and not mere plastids.

of this type are certain white blood corpuscles, or *leukocytes*. Just as these can send out pseudopodia, crawl around in the tissues and engulf and digest bacteria, so certain protozoa known as *amebæ* exhibit exactly the same phenomena. A distinction is made between *lobopodia* which are the digit-like, irregular, soft, and inconstant pseudopodia, and *filopodia*, which are more stiff, less motile, and quite permanent. They are often distributed more or less regularly around the body of certain protozoön organisms, and are therefore known as *actinopodia* (star feet).

2. *Flagella.*—In protozoa these are tapering filaments, broadest at their attached base and ending in a fine tip. Protozoa may have a flagellum at the anterior or at the posterior end; they may have a flagellum at each end and a large number of them distributed entirely around the body. Dobell distinguished four types, depending upon the origin of the flagellum: One in which the flagellum arises directly from the nucleus; a second, in which the flagellar base is united to the nucleus by a connecting filament, the zygoblast; a third, in which the flagellum arises from a basal granule, which is independent of the nucleus, as in herpetomonas; and a fourth, in which the flagellum arises from a special kinetonucleus (*blepharoblast*), as in trypanosoma.

3. *Cilia.*—The cilia of protozoa are similar in type to the cilia lining the epithelial cells of the nasal or uterine mucosa in higher animals. They are generally shorter than flagella, and like them broader at the base and pointed at the distal end. They are peculiar in their motion; first they move rapidly and energetically in one direction, and then they withdraw slowly to the opposite direction. This double motion is repeated more or less continually. Cilia generally show a granule at their basal attached end. They may be distributed uniformly over the whole external surface, limited to one-half or to the ventral surface of the body of the protozoön, or arranged in a single circle around the mouth opening. In some cases cilia are united together to a brush, in other cases they are completely fused to membrane or leaf-like masses, which are then called *membranelles*. The basal granules from which cilia arise have a nuclear derivation, and are called *microsomes*. In many infusoria such kinetic granules are arranged in threads and rows. They form a contractile substance, evidently related to the muscle substance of higher animals, and these primitive muscles are called *myonemes*.

Reproduction.—It has been seen that bacteria which have no differentiated nuclei multiply simply by binary division or fission. The blastomycetes, yeast cells, or budding fungi, which are somewhat higher in phylogenetic development among the simplest forms of vegetable life, possess a nucleus, and when they multiply by spore formation or budding a division of the nucleus always occurs, with a division of the cytoplasm. All animal cells possess nuclei, and nuclear division always occurs in cell reproduction or multiplication. When the nucleus simply divides as a whole without the formation

of a special characteristic arrangement of the chromatic substance the process is called *amitotic division;* when the chromatin arranges itself in a very definite manner in bands or rods, which become equally divided, the process is known as *mitotic division, karyokinesis,* or *karyomitosis.* When this form of division (quite common in protozoa) occurs the kinetic achromatic substance of the nucleus arranges itself in the form of an *achromatic spindle,* with a very small granule or point, the *centrosome,* at either end. The chromatic substance at the same time forms a definite number of threads, bands or rods, which divide by splitting into double the number originally present. They then move in equal numbers toward the centrosomes, so that at the end of division each nucleus possesses again the same number of chromosomes as the original nucleus when it was ready to divide and its membrane began to be dissolved. The number, type, and character of chromosomes is the most constant morphologic part of a cell, and all cells of the same species have the same number of chromosomes which are considered as the carriers of all the elements transmissible by heredity.[1] When two germ cells, however, unite to form a new being the number of chromosomes in each, by a process of *maturation* and by the *expulsion* of chromosomes into *polar bodies,* becomes reduced to one-half, so that the new cell formed by the union of two mature germ cells has not double the original constant number of chromosomes. Several types of cell division occur among protozoa. There is simple binary division with splitting into two equal parts or new cells. This is the type generally found among the cells of higher metazoic animals. Budding as seen in the budding fungi or yeast cells is also encountered, or there may be a splitting up of the cell into a number of smaller daughter cells, each receiving its proportionate share of the nucleus. The nuclear changes in protozoa may closely resemble and be as complicated as the karyokinesis in higher cells, or the granular chromatin may divide as such without solution of the nuclear membrane, which may simply become constricted in the middle and finally be cut in two at this point. The cells of higher animals when dividing generally assume a simple globular form, but in protozoa division is found in fully differentiated cells, for instance, in the flagellate trypanosomes. Starting with the blepharoblast (micronucleus, root of the flagellum) all the structures of the organism, including the flagellum, undulating membrane, macronucleus, and cytoplasm, become split in two sometimes equal, sometimes unequal, masses.

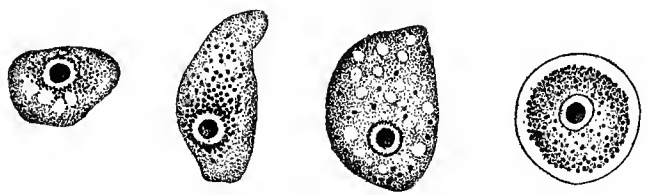
Different Modes of Sexual Reproduction in Protozoa.—In the phylum protozoa a variety of types of reproduction are encountered.

1. *Autogamy, or Automyxis.*—In the fertilization by autogamy, or automyxis, which is widespread among protozoa, there occurs an

[1] It is, however, almost certain that the cytoplasm likewise contains certain constant elements always propagated in multiplication, which are likewise the carriers of hereditary properties of the living substance.

expulsion of chromatin from the nucleus into the cytoplasm. The *cromidia*, or *idiochromidia*, so formed collect in more or less well-defined masses, and these are known as the secondary nuclei, which, however, have no nuclear membranes, but consist simply of seggregated masses of extranuclear chromatin. Two such masses fuse, and this is the process of *syngamic nuclear union, autogamy*, or *automyxis*. It is a self-fertilization and the most primitive type of sexual reproduction

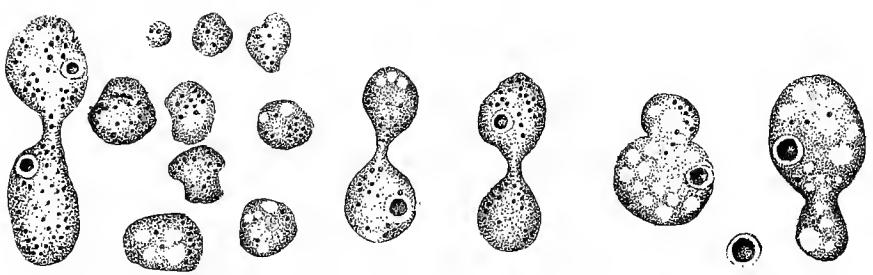
FIG. 182



Amœba limax. Chromidia forming from nucleus and collecting in the cytoplasm prior to encystment. (Calkins.)

in protozoa. It occurs in many species of ameba, and has been studied frequently in Amœba limax. In Entamœba hystolytica, the parasite causing amebic dysentery in man and monkeys, which is not only prevalent in the tropics but also in the southern parts of this country, and even sporadically in the Northern States, Schaudinn and Craig have observed the formation of idiochromidial masses by a fragmentation of the original nucleus. These masses become located at the periphery, are provided with some cytoplasm from the surface

FIG. 183



Amœba limax budding, division, and idiochromidia forming stages. (Calkins.)

as buds, and are finally cut off. In this species of ameba, however, the union of two masses of idiochromidia has not been actually observed. Other species of ameba show a more complicated process of chromidia formation and union (conjugation) between two masses formed. In Amœba proteus, Calkins has observed the following process of autogamous reproduction. There is no formation of diffused idiochromidia, but the secondary conjugating nuclei are

formed directly from chromatin granules within the primary nucleus, which, prior to this stage, had divided repeatedly until about 70 are present. These secondary nuclei next fuse 2 by 2 in the cytoplasm and give rise to spore mother cells (sporoblasts), of which there may be as many as 250 within one parent organism, while at least one of the primary nuclei remains unused and finally degenerates in the cell. In Amœba proteus, therefore, in autogamous fertilization the organism does not form one spore-mother cell (sporoblast), but many.

2. *Endogamy.*—In the mode of fertilization known as endogamy the cell protoplasm breaks down into a number of portions, each one of which receives some nuclear material. After the division of the protoplasm has taken place two of the distinct and separated masses fuse, and around the two united gametes, now known as a copula, a spore wall is formed.

3. *Exogamy.*—When two cells from different ancestors unite and become completely fused the process is known as exogamy. This method is very much like reproduction in higher metazoic animals, where the male and female germ cells become fused to form a new cell. The *copulating* protozoan cells may be perfectly alike (*isogamy*), or they may be different in type (*anisogamy*). In the latter case, in which two cells different in morphology become united, one can be likened to the male and the other to the female germ cells of metazoa. These germ cells of protozoa are called *gametes*. Budding is intimately associated with conjugation, the buds are supplied with chromatin, and they often subsequently become the conjugating gametes. Budding, however, differs from spore formation. In the former case the mother organism which gives off the buds continues to live, while in spore formation the mother cell breaks up into spores and ceases to exist.

Spore Formation.—In some protozoa, particularly in many flagellata, this follows conjugation of two similar cells (isogamy). These similar cells, after having united, form a common cyst, and the protoplasm in the interior of the latter becomes split into a great many very small flagellated organisms. Among the class of protozoa called sporozoa there are two types of spore formation. The spores formed after fertilization are supplied with a firm protecting covering, and they are able to exist outside of the body of the animal in which they are parasitic as mature organisms. The spores formed *asexually* have no such protecting envelope, and cannot live outside of their host. Since the spores formed under such different circumstances differ so much in their biologic properties, they have been distinguished as *sporozoites*, those formed after fertilization, and *merozoites*, those formed in an asexual manner. The former can carry the infection or disease from one host to another; the latter can only carry it from one part of the infected host to another, but they cannot enter the outside world.

The term *sporoblast* designates the mother cell in which the sporo-

zoites have been formed, while *schizont* is the cell which gives rise to the *merozoites*.

Protozoa not Endowed with Eternal Life.—It was formerly believed that the unicellular protozoa could continue dividing indefinitely, that they were endowed with eternal youth and eternal life, and that they did not go through a period of maturity and still less through a period of old age, followed by death. It was first shown by Bütschli, Hertwig and Maupas, and Calkins, by very thorough investigations, that protozoa, even under favorable conditions, after a certain number of generations reach a condition of lowered vitality and depression. In consequence of this they are no longer able to propagate and die unless certain changes occur which lead to the formation of a germ plasm which permits a rejuvenation by sexual reproduction. One of the important changes indicating maturity and the necessity for sexual rejuvenation and reproduction is the formation of the chromidia. This term, as explained, designates the appearance of chromatin granules derived and expelled from the nucleus into the cytoplasm. Schaudinn has shown that the nuclei of conjugating gametes are developed exclusively from such extranuclear chromatin. Mesnil, therefore, proposed to call them *idiochromidia*. The formation of the extranuclear chromidia or idiochromidia in protozoa occurs by nuclear transfusion, by dissolution of nuclear parts, or by nuclear fragmentation.

The facts as to maturity and senility of protozoa show that these low unicellular animal organisms are no more endowed with unlimited youth and unlimited individual life than the higher multicellular animals or metazoa. Both in their body possess only one substance which under the proper conditions of sexual union is endowed with the property of uninterrupted propagation—that is, the *germ plasm*.

Metabolism of Protozoa.—Protozoa can in general only live where there is considerable moisture. They may, however, in the encysted condition and under other circumstances, withstand drying out for a shorter or longer time, and then be like the spores or the vegetative form of bacteria under similar conditions, in a state of suspended animation, from which they may come to life again when the necessary amount of water is supplied. There are a few protozoa, which like plants possess *chlorophyl*, and can derive their food and build up the constituents of their body from very simple compounds forming carbohydrates and proteids from them. However, as an almost invariable rule, protozoa cannot subsist on such simple compounds, but they, like the zoömetazoa, require carbohydrates and proteids in order to supply their demand for growth and multiplication. Most protozoa are supplied with organs of locomotion particularly for the purpose of obtaining food. Such organs of locomotion act by producing in a fluid, currents which bring toward the primitive animal organism other small animal organisms, bacteria, yeasts, and

particles of plants and animals. These may then be engulfed through a mouth organ or they may simply be incorporated into the protozoan protoplasm. Some of the latter, like ameba, possess as organs of locomotion simple protoplasmic processes. These become attached to small food particles, which then become incorporated into the protoplasm by a flowing of the latter around the material intended for food. When the latter are incorporated there is formed in the engulfing protoplasm a food vacuole which secretes either acids or alkalies and a digestive ferment of the peptic or tryptic type. From the foodstuffs certain materials are extracted, others are stored as reserve material (particularly oils and fats), and others again are expelled as waste products. An exchange of gases is likewise kept up by the protozoa, all of which show a more or less high degree of irritability toward chemical and physical influences.

Other protozoa, while existing under the same general laws of metabolism, have by parasitism become adapted to a special mode of life. They exist in the blood serum of higher animals (trypanosomes) or they invade cells of the host (coccidia, plasmodium of malaria of man and birds), and they then generally obtain their food supply by osmotic processes. While the organs of locomotion in the phylogenetic development of the protozoan races have evidently been formed largely with the object of securing the food supply, they have also been used extensively as the basis of the systematic subdivision and classification of the species, etc., of the phylum protozoa.

Classification.—The classification as given in the last edition of Calkins' *Protozoölogy* is followed, but reference is made only to those subdivisions which embrace the parasitic and pathogenic protozoa more fully considered in the following pages.

The subphylum *sarcodina* is defined as protozoa showing no connections with the bacteria, usually of simple structure and characterized mainly by motile organs in the form of changeable protoplasmic processes, the pseudopodia. In this subphylum the subclass *ameba* is of particular interest. It includes the more common forms of rhizopods, with blunt or lobose pseudopodia, which do not anastomose on touching one another. The protoplasmic body may or may not possess a shell.

The subphylum *mastigophora* comprises protozoa in which the kinoplasm is concentrated in the form of one or more vibratile or undulating motile processes called flagella, or in a kinetonucleus which may lie inside or outside of the trophonucleus. This subphylum comprises the very important flagellates, *trypanosoma* and *herpetomonas* and the less important *cercomonas* and *trichomonas*.

The subphylum *infusoria* includes the protozoa in which the motor apparatus is in the form of cilia, either simple or united into membranes, membranelles or cirri. The cilia may be permanent or limited to the young stages. With a micronucleus and a macronucleus, reproduction is effected by simple transverse division or

budding. This subphylum is of little importance from the standpoint of the pathologist. One species only must be considered, namely *Balantidium coli*, and this is of doubtful pathogenic importance.

The subphylum *sporozoa* comprises parasitic protozoa without motile organs, but which are capable of moving from place to place by structural modification of one kind or other. This subphylum is, again, of great importance, because it includes pathogenic *coccidia*, the *malarial organisms* of man and birds, and the *piroplasma* which cause Texas fever in cattle and other animal piroplasmoses.

QUESTIONS

1. Give a definition of the phylum protozoa.
2. Where do they occur?
3. What is their general morphology?
4. Compare the individual variability in bacteria and protozoa.
5. Define the terms spongioplasm, hyaloplasm, ectoplasm, entoplasm.
6. What are plastids and chromatophores?
7. Define the terms macronucleus, micronucleus, trophonucleus, karyogonad, chromatin, kinoplasm, archoplasm.
8. What are the various organs of locomotion found in protozoa?
9. Define the terms lobopodia, filopodia, actinopodia, cilia.
10. Describe the simplest form of reproduction in protozoa.
11. What is meant by mitotic, what by amitotic nuclear division?
12. What is karyomitosis?
13. What are chromosomes? What centrosomes? What the achromatic spindle?
14. What is a blepharoblast?
15. What is autogamy or automyxis?
16. What are chromidia or idiochromidia?
17. What is endogamy?
18. What is exogamy?
19. What are gametes?
20. What is isogamy, what anisogamy?
21. What are sporozoites? What merozoites?
22. What is a sporoblast, what a schizont?
23. Name the different forms of reproduction which occur among protozoa.
24. Discuss the metabolism of protozoa. Can they ever use simple compounds as food?
25. Discuss organs of locomotion with reference to food supply.
26. Discuss intracellular digestion by protozoa.
27. Give the characteristics of the subphylum sarcodina and name some pathogenic microörganisms belonging to this subphylum.
28. Give the same with reference to the subphylum mastigophora.
29. Give the same with reference to the subphylum infusoria.
30. Give the same with reference to the subphylum sporozoa.

CHAPTER LI.

CLASSIFICATION AND MORPHOLOGY OF AMEBA—CULTIVATION—PATHOGENIC AMEBA—ENTERO-HEPATITIS IN TURKEYS—BALANTIDIUM COLI.

AMEBA.

Morphology.—Ameba is a genus of protozoa belonging to the class of rhizopoda of the subphylum sarcodina. The name is derived from a Greek word which means change, indicative of the fact that amebæ are protozoan organisms which, under favorable conditions, constantly change their form. This is brought about by currents in their protoplasm and by the formation of processes extending from the periphery. These processes, which are called *pseudopodia* (false feet), serve as organs of locomotion. If an ameba is suspended in a fluid it is likely to send out short pseudopodia in every direction. As soon as one pseudopodium touches a small solid particle the other pseudopodia are drawn in and the remaining one elongates and draws the whole organism toward the particle to which it has fastened itself. If amebæ are studied on a slide under the microscope it can be seen how they always draw their body along on a pseudopodium extending out from the periphery. The motion is a very peculiar one. It is really not so much a drawing of the protoplasm as a flowing of the latter in the direction of the outstretched pseudopodium, which is generally lobose or lobular in shape. Amebæ generally exhibit a round or oval, more or less vesicular nucleus. The *chromatin* is distributed along the periphery of the nucleus and the interior shows a *central granule*. The protoplasm of amebæ is generally more or less finely granular and very frequently contains one or more *contractile vacuoles* which preferably empty their fluid toward the outside. The *cytoplasm* is generally differentiated into an *entoplasm*, and an often strongly hyalin, *ectoplasm*. Multiplication of amebæ occurs either by fission with an amitotic division of the nucleus, or the ameba may become encysted, forming in its interior a number of young amebæ which, after rupture of the cyst membrane, are set free and grow rapidly. *Spore formation* has also been observed. This is preceded by the expulsion of chromidia or idiochromidia from the nucleus into the cytoplasm. Small masses of chromatin reach the periphery, are extruded from it, and finally cut off with a small amount of protoplasm. From these spores young amebæ are developed. Amebæ are found in the outside world and in moist soil, where they lead a purely saprophytic existence, or they may be

found in the intestines of a great variety of animals, where they lead a parasitic, but as a rule perfectly harmless, life.

Microscopic Study.—The study of ameba is to be undertaken on fresh preparations in the live state, and it is then best to use the fluid in which they naturally occur. Saprophytic amebæ are, therefore, best studied in the water in which they are found; parasitic amebæ can be studied in feces or in scrapings from the intestines, perhaps mixed with a small amount of physiologic salt solution. Of such fluids containing saprophytic or parasitic amebæ a *hanging drop* may be made or a drop of the fluid is placed on a slide and covered with a cover-glass. If the cover-glass be used it is well to supply it with four very short wax feet, so that the weight of the cover does not compress the amebæ, as they are much more sensitive to insults than bacteria, and a small degree of pressure may injure them. When amebæ in semisolid or semifluid feces are studied it is not necessary to supply the cover-glass with wax feet, because such material generally contains a sufficient number of undigested particles to furnish a support for the cover-slip. When parasitic intestinal amebæ are studied in the fresh state it is well *to warm the slide and cover-glass*, because such amebæ lose their motility as soon as they are materially cooled. Care should be taken not to heat the slide and cover-glass too much, as otherwise the amebæ go into a condition of "heat rigor," and become likewise immobile. The best method of warming the slide and cover-slip properly is to immerse them for some time in water a little warmer than body temperature (about 40° C.).

Staining Properties.—Amebæ should also be studied in stained preparations. The simplest method of studying stained amebæ consists in the preparation of thin smears or spreads on cover-glasses. For the study of intestinal amebæ, Craig recommends Oliver's modification of Wright's staining method. The spread is made on the slide and is allowed to become air dry, and then a few drops of Wright's stain are poured on the slide. The stain, being dissolved in methyl alcohol, fixes at the same time that it dyes. The stain is allowed to act for five minutes, then enough distilled water is added to cause a slight metallic scum to appear on the surface. The dilute stain then remains on the slide for ten minutes longer and the preparation is finally well washed in distilled water and dried between filter paper. The slide is not mounted in Canada balsam, but is examined directly with the oil-immersion lens. If after examination the specimen is to be preserved the immersion oil is washed off with xylol. Walker recommends the chloride of iron hematoxylin method of Mallory for staining amebæ in slide and cover-glass preparations. The smears after being air dry must first be fixed a short time in Zenker's solution,[1] then washed successively in water,

[1] Zenker's solution is composed of bichloride of mercury, 5 grams; bichromate of potash, 2.5 grams; sulphate of sodium, 1 gram; glacial acetic acid, 5 c.c.; and water enough to make 100 c.c.

iodine-alcohol, and pure alcohol, and dried. The steps of the iron hematoxylin method are the following:

1. Stain smears for three to five minutes in a 10 per cent. watery solution of ferric chloride.

2. Drain and blot the cover-glass, then pour over it a few drops of a freshly prepared 1 per cent. aqueous solution of hematoxylin. If all the hematoxylin is precipitated by the excess of ferric chloride, pour off the solution and add a fresh supply. In three to five minutes the sections will be colored a dark bluish black.

3. Wash in water.

4. Decolorize and differentiate in a $\frac{1}{4}$ per cent. aqueous solution of ferric chloride. The cover-glass must be kept constantly moving in the solution. The differentiation will be complete in a few seconds to several minutes.

5. Wash in water, dry, and examine.

If the differentiation is not sufficient the preparation must again be washed in the $\frac{1}{4}$ per cent. solution of ferric chloride. Mallory states that the principal point in this method is first to stain very deeply and then to differentiate properly. The nuclei of amebæ stain sharply with this method.

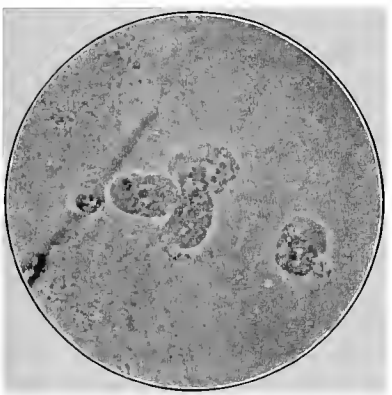
Cultural Characteristics.—Attempts to cultivate amebæ had been made for a number of years, but not much progress was made until Musgrave and Clegg devised a method which is now generally used, either according to the original recommendation or with some slight modification. The principle of this method consists in preparing a culture soil, comparatively poor for bacteria, which will enable them to thrive only moderately, but sufficiently to serve as food for the amebæ, which require proteids for their metabolism and cannot utilize simple compounds like plants. Musgrave and Clegg's medium consists of:

Agar . .	. 20 gr.
Sodium chloride .	0.3 to 0.5 gr.
Extract of beef	0.3 to 0.5 gr.
Water .	. . . 1000 c.c.

This is prepared in the same manner as ordinary agar and made 1 per cent. alkaline to phenolphthalein. The medium is sterilized in tubes and from these Petri dishes are filled in the ordinary manner, but, of course, without inoculating anything into the sterile, melted agar before it is poured out into the lower plate of the Petri dish. The agar is there allowed to solidify. If amebæ are to be cultivated from water or from water mixed with vegetable material, 100 to 500 c.c. of the fluid are placed in a flask and 0.5 to 1 c.c. of ordinary nutrient bouillon is added for each 100 c.c. of amebæ-containing fluid. The flasks are then set aside and kept at room temperature for from twenty-four to seventy-two hours, when a few loopful of fluid may be removed from the surface, spread on a slide and examined fresh for the presence

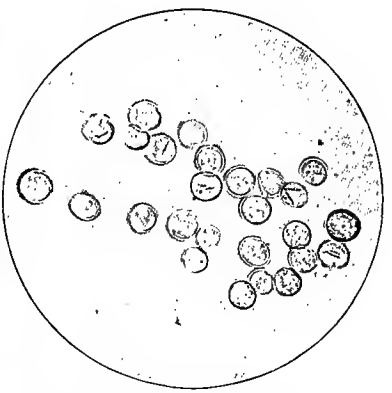
of amebæ. If microscopic examination reveals amebæ, one or more loopfuls of the surface fluid containing them is streaked over the surface of the set agar in the Petri dishes. In the course of from six to forty-eight hours the plates are to be examined under a low power of the microscope in the same manner as for bacterial colonies. If the amebæ have multiplied, at they usually do, they can be recognized under the low power of the microscope as highly refractive bodies. From such plates others can be inoculated by making transplants and again streaking the agar on the surface. In trying to obtain a growth of intestinal amebæ it is necessary to streak small particles of feces, best some mucous flocculi picked up with the platinum loop over agar plates, because such parasitic amebæ do not multiply as easily in fluid as do saprophytic amebæ from ordinary outside sources.

FIG. 184



Ameba from a case of tropical dysentery in man. Twelve hours' culture. (Musgrave and Clegg.)

FIG. 185



Encysted forms of an ameba from an old culture. (Musgrave and Clegg.)

When amebæ grow after such an inoculation several kinds may be present in addition to a variety of bacteria. It is, however, necessary to isolate one kind of ameba and to cultivate it with one known kind of bacterium. No one has ever succeeded in cultivating amebæ alone in absolutely pure culture, because they evidently need for their nutrition live, unchanged proteid material. The best that has so far been accomplished has been to obtain amebæ in symbiotic community with one known species of bacterium. Such a culture has been called by Frosch "*a mixed pure culture of amebæ.*" Musgrave and Clegg succeeded in getting such pure cultures in a manner described by them as follows:

"Select a plate culture on which the parasites are well distributed and after removing the cover, place the plate with the open side up on the stage of the microscope. By searching the edges of the growth

with a low-power objective, places will be found where the ameba are some distance apart. After locating a satisfactory parasite, which should be one on the surface of the medium, as practically all of them are, and having determined that there are no others in the field, either on the surface or at a depth, swing a perfectly dry and clean high-power lens in place and gently lower it until the entire surface is in contact with the medium. Raise the lens quickly, swing in the low-power objective and determine whether the ameba is still present or has been picked up. If it has been picked up, which after some practice may be done two or three times out of five, the lens to which the ameba adheres is removed, and, by gently rubbing its surface over that of a plate containing the hardened medium the organism may be transferred. In this manner a pure culture, so far as amebæ are concerned, may be obtained. That only one ameba has been carried over by this method may still farther be verified by examining with a low-power objective the closed inverted plate on which it has been inoculated. Another useful result of a careful application of this method is the aid it gives in obtaining pure cultures of an ameba and of a single bacterium. The lens, of course, picks up the bacteria from a small field immediately surrounding the ameba; and as such isolated ameba is often surrounded by one kind of bacterium only, with the aid of a careful bacteriologic technique the pure cultures desired may sometimes be obtained in this manner." Musgrave and Clegg have used the preceding method in most cases and have found it satisfactory. It would be possible to obtain the desired results more easily and with greater constancy by means of Unna's bacterial harpoon or a specially constructed lens, with a short adjustable focus and a cup-shaped extremity, like the marking arrangement which has been suggested for locating special fields in permanent preparations.

Parasitic amebæ obtained in cultures on plates growing there in symbiosis with bacteria will not grow with any and all bacteria, but they are quite selective, at least, at first, and it is, therefore, necessary to obtain from the mixed culture on the first plates all bacteria present in pure cultures. After these have been obtained and a number of individual amebæ have been picked out by the method described above and transferred to fresh plates such planted amebæ are surrounded by several concentric streaks of bacteria obtained from a pure culture. If the necessary precautions have been taken, most amebæ, as they multiply, will quite generally spread rapidly over the plate, and in passing through the rings of growing bacteria they will lose the organisms with which they started and take up those forming the rings. In from twenty-four to seventy-two hours the protozoa will have passed one or more of the rings, and from such locations they may be taken for transplanting. It sometimes happens that they appear on the first plate in pure cultures with the desired organism, but usually one or more transplants to the same

medium are necessary before this end is reached. The further inoculations are made with amebæ obtained from outside the largest ring on the next preceding culture.

This method is simple in execution, and the entire process may be watched under the microscope by inverting the plate and using a low power, according to the method employed in studying colonies of bacteria. With a low power the wanderings of the amebæ and even their multiplication can be kept under observation.

The ring-shaped smear of bacteria has several advantages over one covering the entire surface of the plate. In the first place amebæ develop more rapidly by its use, and secondly, they lose the original organisms much more rapidly than when moving constantly over a bacterial substratum.

Another feature which commends this method is the facility with which it lends itself to a determination of the symbiotic value of a given organism. If such an organism for any reason is not satisfactory to the amebæ they will not mix with or cross the bacterial rings. In some instances, where the organism is particularly unfavorable, the amebæ, after wandering up to the inner margin of the first ring, encyst, and no further progress is made. On the other hand, where the antipathy is less marked, the progress is simply delayed until the bacteria carried over in inoculating the amebæ have mixed with or crossed the ring, whereupon the amebæ follow them.

When amebæ have been isolated and grown in pure culture with a satisfactory symbiotic organism it is sometimes difficult to transfer them to another. This is best overcome by first cultivating the protozoa for a short time on a mixed culture of the two organisms and then isolating them with the desired one by the use of the method described. Even by this means success is often doubtful and sometimes impossible of attainment.

Among the bacteria with which amebæ have entered into symbiotic community as reported by various observers are the spirillum of Asiatic cholera, typhoid bacillus, Bacillus coli, Bacilli fluorescens liquefaciens and non-liquefaciens, Staphylococcus pyogenes aureus, Bacillus pyocyaneus, Bacillus ruber, spirillum of Metchnikoff, various other bacteria, and also yeast cells.

Walker, starting out from pure mixed cultures, according to the above method, has modified it in such a manner that he could study the whole development under the microscope. He calls his device the "*hanging-plate culture*," and prepares it as follows: A thin $\frac{7}{8}$ inch cover-glass is flamed and placed under a flamed watch-glass. With a large platinum loop a large loopful of melted sterile agar is transferred to the sterile cover and spread in a uniform, thin, and circumscribed layer. This film of agar will solidify almost instantly, and it is at once inoculated from a pure mixed ameba culture. The cover-glass culture is then inverted over a concave slide which has been flamed, cooled, and rimmed with vaselin. On such cover-glasses

amebæ multiply as freely as on Petri dishes. The film of agar medium is thin enough to permit the use of a 2 mm. oil-immersion lens.

Pathogenic Amebæ.—Amebæ have frequently been found in the intestinal tract of man and the lower animals. They were perhaps first seen in 1859 by Lamble and demonstrated beyond a doubt by Loesch in 1875, who found them in the discharges of a patient suffering from chronic dysentery. Loesch called the organism *Amœba coli*, and claimed that he was able to produce dysentery in dogs by the injection of the feces containing them. Amebæ as the possible cause of disease in man or animals, however, did not attract much attention until Robert Koch, while studying Asiatic cholera in Egypt, found them in the tissues of the intestines of three persons who had died from chronic dysentery. Koch's observations stimulated the work of Kartulis, who found amebæ in the stools of 150 sufferers from dysentery, and who published his investigations in 1886. Observations in this country were then made by Osler, Musser, Stengel, Dock, Councilman and Lafleur, Harris and others. While, for a number of years, there has been no doubt that amebæ are found in certain dysenteries in man, their role in the production of this disease has been again and again in doubt, as it has been shown that they occur in all forms of diarrhea and frequently in the stools of perfectly healthy persons and in the intestines of many species of animals which are in a normal condition of health. Walker recently described 37 species of amebæ (including several previously undescribed species) in the intestines of man, the horse, pig, dog, cat, rabbit, guinea-pig, rat, house mouse, white mouse, etc., the great majority of which, beyond doubt, are perfectly harmless commensales in the intestines of their host. The question of the pathogenicity of amebæ in man has been much clouded because most observers had not learned to differentiate a harmless commensale from a truly pathogenic organism. Two observers in particular, Schaudinn and Craig, however, have clearly shown that one common non-pathogenic ameba and one pathogenic species are really found in the intestines of man. Schaudinn was the first to describe these two types definitely. Craig made some early observations independent of Schaudinn, and later confirmed all of Schaudinn's observations as to the fundamental difference between the two types. Schaudinn named the harmless commensale in the intestines of man *Entamœba coli*, and the pathogenic organism which is the cause of so-called amebic dysentery *Entamœba hystolytica* (histolytica, tissue dissolving).

Entamœba Coli.—This harmless commensale was found by Schaudinn in the feces obtained after purging in 50 per cent. of healthy persons examined in West Prussia; in 20 per cent. of persons examined in Berlin, and in 66 per cent. of persons examined along the shores of the Adriatic. Craig found this organism in 65 per cent. of healthy American soldiers examined in San Francisco; Ashburn and Craig, in 71 per cent. of healthy American soldiers in Manila, and Vedder,

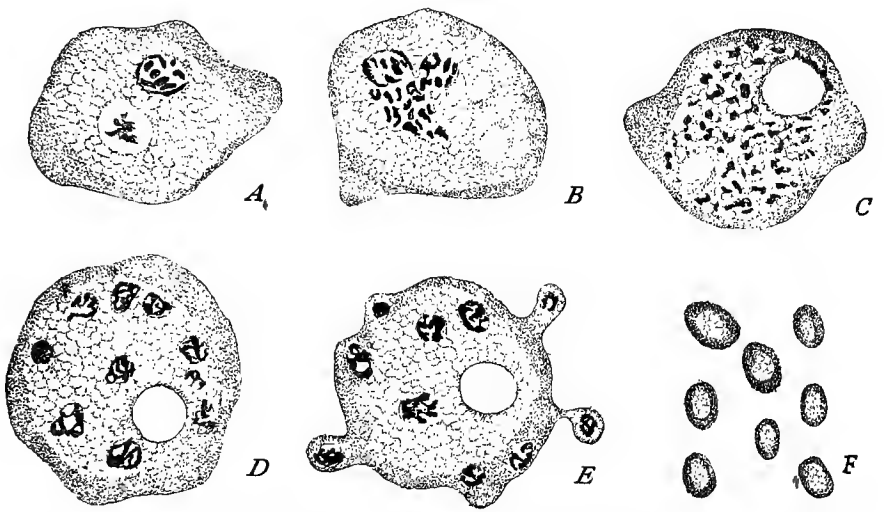
in 50 per cent. of American soldiers in Manila and 72 per cent. of Philippino scouts. In order to find Amœba coli in the stools of healthy persons it is practically always necessary to administer a large dose of salts, such as sulphate of magnesium or sodium. The discussion as to pathogenic and non-pathogenic amebæ has been of the greatest importance, since it makes a good deal of difference whether all amebæ may occasionally become pathogenic, or whether among them, as among bacteria, certain definite types produce specific diseases, while others are perfectly harmless and non-pathogenic. In animals the same conditions, of course, prevail, and in them, apparently, as in man, most amebæ inhabit the intestines as harmless commensales, but likewise one species has already been found which evidently is a very dangerous pathogenic parasite.

Entamœba coli, as described by Schaudinn, Craig, and others, consists of a mass of protoplasm, containing a well-defined nucleus and generally one or more nucleoli. Sometimes a non-contractile vacuole is present, but rarely, if ever, more than one vacuole. The differentiation between the entoplasm and ectoplasm is very faint; the organism is very sluggishly motile. Reproduction under favorable conditions occurs by simple division, under unfavorable conditions after encystation followed by the formation of *eight daughter cells in the cyst*. The daughter cells after solution or rupture of the cyst membrane are set free and develop into young amebæ. Entamœba coli varies in size between 8 to 50 micra, generally between 25 to 30 micra. When not in motion it is spherical in shape. Its pseudopodia are rounded or lobose, never sharply pointed. It is of a dull grayish color, and takes up red blood corpuscles very rarely even if they are present in the feces. Bacteria are often found in the finely granular protoplasm. The nucleus, 5 to 8 micra in diameter, is generally situated a little to one side of the centre. It has a thick, easily seen nuclear membrane and possesses a large amount of chromatin. In stained specimens the ectoplasm dyes very dimly, the entoplasm intensely. The latter is composed of well-defined granules, among which engulfed bacteria can generally be seen; the chromatin of the nucleus is shown as short strands or round granules. The encysted forms do not take the stain on account of their firm capsule. When encystation occurs the organism becomes perfectly motionless and develops from its spherical periphery a highly refractive hyaline membrane which finally acquires a double outline or contour and appears irregularly striated. During the formation of the cyst wall the organism apparently contracts and loses about one-third of the diameter it had when encystation began. The protoplasm in the cyst becomes hyalin, the nucleus breaks up, and eight daughter nuclei are formed, around these cytoplasm is distributed, and in this manner eight young cells originate in the interior of the cyst.

Entamœba Hystolytica.—This is the pathogenic type of ameba parasitic in the intestines of man, and is the cause of *chronic amebic*

dysentery. It penetrates into the intestinal mucosa and submucosa, and in this manner produces ulceration. It can even be carried to the liver and there produce abscess. Amebic dysentery is most prevalent in the tropics, but it is also found in the United States, particularly in the Southern States. The organism consists of a mass of protoplasm, contains a nucleus, and generally several non-contractile vacuoles. It is round when at rest. It is generally larger than Entamœba coli. Craig gives its average diameter as 35 micra, and says that he has seen individuals as large as 60 to 70 micra. In full-grown individuals the differentiation into a granular entoplasm and a hyaline ectoplasm is very marked. The latter forms a consider-

FIG. 186



Entamœba hystolytica. (After Craig.) *A*, organism showing rods and granules of chromatin in the nucleus, vacuole with some stained substance, and dense ectoplasm; *B*, the chromatin of the nucleus passing into the cell plasm, where it is distributed as chromidia, shown in *C*; *D*, aggregation of chromidia to form secondary nuclei; *E*, "spore formation" by budding; *F*, spores of Entamœba histolytica as seen in feces.

able portion of the entire cytoplasm; it is highly refractive and glass-like in appearance. It can generally be seen at its best when the organisms are examined in a warm, fresh stool. Here it is generally very lively motile, much more so than Entamœba coli. When pseudopodia are formed a rapid or also more slow outflow of the hyaline ectoplasm takes place, and the pseudopodium is first formed of it alone, later the granular entoplasm also flows in. Schaudinn and Juergens believe that the power of the Entamœba hystolytica to penetrate into tissues depends primarily upon the evidently very firm tenacious ectoplasm. The nucleus is not easily distinguishable in the live unstained condition, and it contains a small amount of chromatin only. The pathogenic entameba when found in bloody stools often

contains several and sometimes many red blood corpuscles. These are digested in the interior of the parasite, and its protoplasm and the fluid in the vacuoles frequently show a slight greenish tinge. If stained with Wright's stain the ectoplasm stains very intensely, while the entoplasm stains lightly. Entamœba coli behaves in exactly an opposite manner. The nucleus of Entamœba hystolytica stains poorly on account of the scanty amount of chromatin. Reproduction occurs by division and budding with spore formation. The latter appears to occur in the intestines of man when conditions become unfavorable to the organism and when it becomes advantageous for it to assume the more resistant spore form. Then the nucleus expels and distributes most of its chromatin into the cytoplasm, the expelled chromatin collects into small masses, and these reach the ectoplasm, where they become protruded with some cytoplasm, beyond the periphery of the main body, and are finally cut off from the mother cell entirely.

Human feces containing Entamœba hystolytica, when injected into the rectum of kittens, produce typical, generally fatal attacks of amebic dysentery. Upon postmortem examination the characteristic ulcerative changes of the disease are found in the intestines.

From a study of various cultures of amebæ, Walker came to the conclusion that Schaudinn's observation on the differences between Entamœba coli and Entamœba hystolytica were incorrect; but, as Craig properly remarks, Walker studied only a very few cases of human dysentery, and confined his observations to a few cultures of intestinal amebæ from human sources. The characteristic reproduction by budding in Entamœba hystolytica, however, cannot be observed in artificial cultures, but must be studied under the natural conditions in which this pathogenic organism it found in the intestines and discharges of man.

New Species of Pathogenic Amebæ.—Several new species of amebæ pathogenic to man have recently been described by observers working on cases of chronic tropical dysentery in different parts of the world.

Viereck studied the stools of 62 cases of dysentery from Africa, Europe, and South America. He found living amebæ in 37 cases, and bodies which he thought were amebæ in 17 cases. In 2 cases he encountered an ameba which resembled Entamœba coli more than it did Entamœba hystolytica, but it formed four cysts instead of eight. It produced dysentery in cats. Viereck also found this ameba in non-dysenteric stools, and suggested that it might be a variety of Entamœba coli. He called it *Entamœba tetragena.*

Hartmann, almost simultaneously with Viereck, described an ameba which was found to be identical with Entamœba tetragena. This organism was found only in cases of dysentery, and it produced typical ulcerating dysentery in cats. As a rule, it is not as pathogenic for cats as the Entamœba histolytica. In all cases from Africa and South America which Hartmann examined he found Entamœba tetragena.

Wemer confirmed Viereck's and Hartmann's findings. In one case he observed an ameba which differed from Entamœba tetragena and also differed somewhat from, but closely resembled, Entamœba hystolytica. He attempted to cultivate the pathogenic ameba, but failed. He was able to get amebæ to grow, but always found that they were the non-pathogenic forms, and considers them to have been present with the pathogenic amebæ in the material which he used in making his cultures. He is of the opinion that pathogenic amebæ have so far not been cultivated, and that, therefore, all studies of amebæ from culture have been of non-pathogenic amebæ.

Noc studied the amebæ from the drinking water in Saigon, also the amebæ from the stools of cases of dysentery and from the pus of liver abscesses originating in Saigon. He was able to cultivate the amebæ from these sources, and found that he had the same organism in the drinking water, the stools of cases of dysentery, the dysenteric ulcers of the intestines, and the pus of liver abscesses. This ameba closely resembled Entamœba hystolytica, but differed from it in being rich in nuclear chromatin and in forming large polymorphous cysts. It also differed from Entamœba tetragena and Entamœba coli.

Infectious Enterohepatitis in Turkeys.—Enterohepatitis in turkeys is an infectious disease apparently due to a pathogenic protozoön called Amœba meleagridis by Theobald Smith, its discoverer. Cushman, of Rhode Island, in 1893, noticed a peculiar disease among turkeys which has since been found in various other, particularly Eastern, States. The affection is characterized by diarrhea and generally progressive emaciation, and a dark discolorization of the comb, wattles, and the skin of the head, from which the common name *black-head of turkeys* is derived. The disease frequently attacks young birds, and it may take a more acute or a markedly chronic course. The most important pathologic changes are the following: The ceca of the birds show a thickening of the wall and a superficial and even deep destruction of the mucosa and submucosa. The thickening may be uniform, or it may be present in circumscribed places only. The changes are generally most marked near the blind ends of the intestinal pouches; sometimes the cecum only is diseased while the other part is not changed. In the early stages the adenoid tissue between the pouches and in the submucosa becomes much increased. With the extension of the disease much of the mucous membrane may become destroyed by ulcerative and desquamative processes, and fibrinous material is deposited on the denuded intestinal surface of the affected intestines. In the majority of cases secondary lesions are found in the liver, which, according to the description of Smith and Moore, is enlarged to perhaps twice its normal size. On the surface are seen roundish discolored spots, sharply differentiated from the rest of the tissue. They vary from 3 to 15 mm. in diameter, and may be lemon-yellow, neutral-gray, ochre-yellow, or of a mottled brownish

color. They are not elevated but rather depressed, and contain necrotic material in their interior. Such foci are also found in the depth of the liver tissue, not reaching up to the surface. In these areas, as in the cecal pouches, large numbers of amebæ are found. According to Smith's observations these are very numerous in the affected tissues in recent cases or when the disease is at its height. They disappear, however, from areas where degenerative and necrotic changes are much advanced. The most frequent appearance presented is that of round homogeneous bodies with a sharply defined single contour. Within the parasites situated a short distance away from the centre is a group of minute granules probably representing nuclear chromatin. The Amœba meleagridis is generally between 8 to 10 micra in diameter, sometimes between 12 to 14 micra. The tissue reaction against the invading protozoa leads to the formation of giant cells, and the parasites are often seen in their protoplasm. This, however, is a process of phagocytosis on the part of the tissue cells, because the Amœba meleagridis is evidently not an intracellular parasite, but it penetrates between the cells, which it destroys by the intercellular invasion. The life cycle of this organism has not yet been studied.

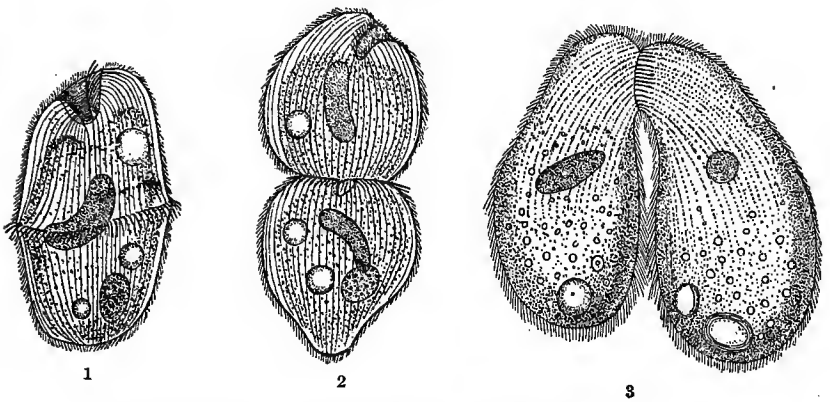
BALANTIDIUM COLI.

The subphylum infusoria is not of great importance as far as microörganisms pathogenic to higher animals and men are concerned. From what is known today, there is one infusorium which may produce disease in man and which is frequently a parasite in the intestines of the hog, from which it probably occasionally invades the large intestines of man. This infusorium is the Balantidium coli. It can generally be obtained for the purpose of study in the intestinal contents of the hog. The organisms belong to the subphylum infusoria, class ciliata, order heterotrichida, family bursaridæ. Infusoria possess cilia as a motor apparatus and a macronucleus and micronucleus. The members of the family to which balantidium belongs generally have a short, pocket-like body. Their chief characteristic is a *peristome*, which is not a mere furrow, but a broad, triangular, deeply excavated area, which ends at a point at the mouth.

The body of Balantidium is oval or egg-shaped. It possesses a short gutter or funnel-shaped peristome—which is continued into a short esophagus. The body is externally lined by a fine skin or pellicula, under which an alveolar ectoplasm is found. The whole exterior is covered by fine cilia. The entoplasm is cloudy and contains droplets of mucus and fat. In it two contractile vacuoles are also situated. The anus of the infusorium is indicated by a prominence in the protoplasm. It is, however, always fused except during the act of expelling waste material. The macronucleus is kidney- or bean-shaped, and contains much chromatic material. The micro-

nucleus is small, round, and vesicular. The size of Balantidium coli varies between 60 to 100 micra, its width between 50 to 70 micra. Reproduction occurs by division, budding, and conjugation. In division the macronucleus divides amitotically, the micronucleus mitotically. Leuckard has described encystation of the parasite. If this is to occur the cilia are gradually lost, except a few near the mouth, and the body finally becomes perfectly round and surrounds itself with a heavy capsule. Encystation has also been noticed after conjugation. Very probably the infection is transmitted from one host to another in the encysted stage of the parasite. The organism, according to a monograph of Strong, has been found in about 150 cases in man, generally in cases of obstinate diarrhea. In some of

FIG. 187



Balantidium coli: 1, 2, stages of division; 3, conjugation. (After Leuckart.)

the observed cases death followed. Most authors look upon balantidium as a harmless commensale of the hog and occasionally man, but some, like Strong, believe that it may be the cause of diarrheal intestinal disturbance and the ulcerations accompanying it. Strong and others have found the balantidium in the intestinal tissues, and there is no doubt that the organism engulfs and digests the blood corpuscles of its host. The author has seen a case of intense balantidium infection in a Filippino, but since the infusorium was associated with an uncinaria infection, it was impossible to decide to which of the two the pathologic disturbance was mostly due. Brooks has described an epidemic among the orang-utangs of the New York Zoölogical Garden, in which balantidium was found in large numbers in the stools. Several of the animals died and the postmortem examinations showed ulcerations in the intestines and balantidium in the tissues.

QUESTIONS.

1. Give the proper classification of ameba.
2. What does the term ameba signify?
3. What is a pseudopodium? What a lobose pseudopodium?
4. Describe the general morphology of amebæ.
5. What methods of reproduction have been observed?
6. What is meant by endoplasm, ectoplasm, chromidia formation?
7. How can amebæ be studied in the live state?
8. Describe the two staining methods recommended for the study of amebæ.
9. Describe the culture medium used in cultivating amebæ.
10. What is a pure-mixed culture of amebæ?
11. What is the procedure for obtaining a growth of amebæ: (*a*) in the case of saprophytic amebæ, (*b*) in the case of parasitic amebæ?
12. Describe in detail the subsequent steps to get a pure-mixed culture of amebæ.
13. Do amebæ enter into symbiotic community with all bacteria?
14. What is the "hanging plate culture" method of Walker?
15. Are all parasitic amebæ pathogenic? Name some pathogenic amebæ.
16. Describe the morphology of Entamœba coli.
17. Describe the morphology of Entamœba hystolytica.
18. Give the main differential morphologic and biologic features of Entamœba coli and Entamœba hystolytica.
19. Is Entamœba coli ever found in the intestines of healthy man? If so, what is the procedure for finding it?
20. Name some other amebæ (besides Entamœba hystolytica) pathogenic to man.
21. What is enterohepatitis in turkeys? What is its common name?
22. In what organs and structures are the main pathologic lesions of this disease found?
23. Describe the chief pathologic lesions.
24. Name and describe the organism causing infectious enterohepatitis in turkeys.
25. What kind of an organism is Balantidium coli?
26. Where generally found? Does it ever infect man?
27. Is it ever pathogenic to man or animals? What reasons are there to believe that it is pathogenic?

CHAPTER LII.

TRYPANOSOMES AND TRYPANOSOMIASES—CERCOMONAS—TRICHOMONAS—HERPETOMONAS.

TRYPANOSOMES.

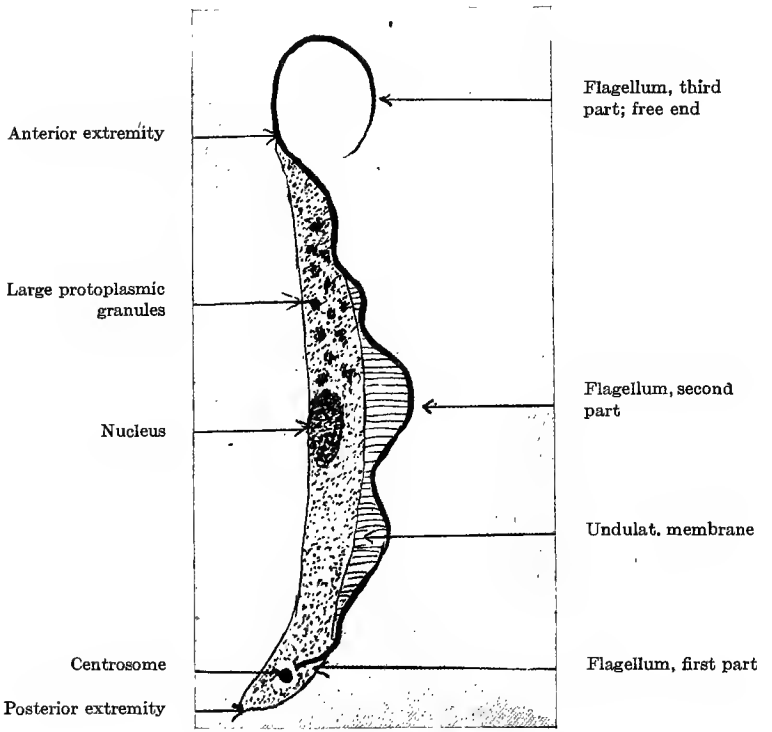
Historical.—Trypanosomes were first seen by Valentine in 1841 in the blood of trout, and in the following two years they were found by several observers in the blood of a number of species of frogs. The first trypanosomes in a mammal was discovered by Lewis in India, in 1878, in the blood of the rat. Two years later Evans, chief veterinarian of the English Army in India, discovered trypanosomes in the blood of horses, camels, and other animals sick with the affection known as *surra,* and he expressed the opinion that these blood parasites were the cause of the disease. This claim was, however, at that time not widely accepted and the study of trypanosomes as an etiologic factor of disease was not extensively undertaken until Bruce, in 1894, had discovered trypanosomes as the cause of *nagana* of horses and cattle in Africa. Since that time these flagellata have been discovered in a number of animal diseases, and today their great importance in veterinary and human pathology is well established.

Classification and Morphology.—Trypanosomes belong to the protozoan subphylum mastigophora (whip carriers), to the first class (zoömastigophora) in which animal characteristics are predominant, and they form the fourth order of this class. They are defined by Calkins as follows: "Organisms of elongated, usually pointed form, and a parasitic mode of life, with one or two flagella arising from a special motor nucleus, and with an undulating membrane provided with myonemes running from the kinetonucleus to the extremity of the cell; one of the flagella is attached to the edge of this membrane throughout its length, and may terminate with the membrane or be continued beyond the body as a free lash."

Trypanosomes generally have an elongated spindle-, lancet-, or eel-shaped protoplasmic body; sometimes the spindle is almost as wide as it is long. This, however, is only exceptionally the case, and the student first familiarizing himself with trypanosomes, particularly those in higher vertebrates, will do well to remember them as little, rather slender, eel-shaped bodies of the size of an involuntary muscle cell of the non-pregnant mammalian uterus. Their protoplasm shows two chromatic or nuclear masses. One of them, as a rule, placed at or near the centre is a comparatively large, finely granular body, called

the nucleus proper, the *tropho-* or the *macronucleus;* the other one, quite small and dot-like, is known as the *micronucleus,*[1] or, more properly, the *centrosome*, or *blepharoblast*, and it is generally found at the posterior end of the microörganism. From the centrosome starts a thin, folded membrane, the undulating membrane, it has a thickened border which runs out into a free whip-like filament called the flagellum. The latter is composed of three parts, the root which

FIG. 188



Morphology of Trypanosoma Brucei (schematic).

arises from the blepharoblast and extends in the protoplasmic body as far as the undulating membrane, the second portion which runs along the free border of the latter, and the third portion which is the filamentous free end. The undulating membrane and the flagellum form the organs of locomotion of the trypanosome, and they enable

[1] Calkins says: "The terms macronucleus and micronucleus are frequently used to designate the *trophonuclei* and *kinetonuclei* of these flagellates (trypanosomes), but this use of the term micronucleus is greatly to be deplored, since the kinetonucleus has absolutely no analogy with the micronucleus of infusoria, and the binucleate condition of the trypanosomes is to be explained upon other grounds than that of the ciliates."

it to move about freely and actively in the body fluids where is has its usual habitat.

While the protoplasm of the trypanosome shows a certain amount of contractility, its motion is almost exclusively due to the undulating membrane and the flagellum, and it is in the direction to which the free whip-like filament points that the organism moves. For this reason the end from which the free flagellum projects is generally called the anterior extremity. A few species of trypanosomes have a free flagellum at each end, but this is an exceptional occurrence among these flagellata. In addition to the structures described the protoplasm of trypanosomes shows larger, smaller, and very minute granules, which exhibit special staining properties, and which are often arranged in rows or striæ; occasionally clear open vacuoles are seen in their posterior part.

From the foregoing description it may be inferred that these animal microörganisms, though unicellular and parasitic, show a high degree of differentiation into a number of morphologic components. When trypanosomes multiply they do so by a longitudinal splitting. The micro- and macronucleus divide first, and the process of splitting then generally extends to the protoplasm, the undulating membrane, and the flagellum. The macronucleus, however, never, like the nuclei of most of the cells of higher plants and animals, shows the formation of karyokinetic figures, but it divides amitotically, that is, by direct division.

Habitat.—The usual habitat of the trypanosomes is the blood of vertebrate animals. In certain diseases they are also found in the lymph and in the cerebrospinal fluid. They are, as far as is known, strict parasites which can only exist and multiply within another living host. There is no evidence that trypanosomes are ever found free in the outside world. Unlike the hemosporidia of malaria and the piroplasma of Texas fever, trypanosomes do not invade and live inside the red blood corpuscles, nor do they engulf into their own protoplasm and digest the erythrocytes of the blood of their host, as, for instance, the amebæ of dysentery do. They simply float about in the blood plasma and derive food for existence and multiplication by processes of osmosis. While trypanosomes have been found in the blood of many types of vertebrate animals, such as batrachians, reptiles, fishes, birds, and mammals, nothing would be more erroneous than to consider all of them dangerous disease-producing parasites. Quite to the contrary, most trypanosomes so far discovered are harmless blood parasites and apparently no more dangerous to their host than many of the bacteria which inhabit the integument, the gastro-intestinal and the genito-urinary tract of the higher animals and man as commensales.

Method of Examination in Trypanosoma Infection.—Demonstration of trypanosomes in infected animals is, as a rule, a very easy matter, although they are sometimes so scanty that it may be necessary to

concentrate them by preliminary centrifuging. Frequently, however, it is only necessary to obtain a drop of blood, for instance, from the ear of a larger animal or from the tip of the tail of a rat, or mouse, allow it to fall on a clean slide and cover it with a cover-glass. This simple preparation should be examined at once with the microscope. The first search can be made with a low-power lens, 16 mm. or two-thirds inch focus. With this magnification a peculiar agitation among the red blood corpuscles can frequently be seen in a part of the field. If this spot is placed in the centre the very characteristic micro-organisms can generally be seen either with a high-power dry or with an oil-immersion lens. In fresh preparations trypanosomes easily betray their presence even to the tyro by their shooting, darting, or spiral motions in the blood, and, in some trypanosome infections, as, for instance, in *dourine* in horses, in the juice expressed from the patchy infiltrations of the skin or the inguinal glands, and in *sleeping sickness* in man in the centrifuged cerebrospinal fluid. If the morphology of the pathogenic trypanosomes is to be studied in detail, dry stained preparations are necessary. The most useful stains are generally a combination of eosin and methylene blue as found in the stains of Romanowski, Leishman, and Wright. The last named, in particular, is very satisfactory, because its use is very simple. It is only necessary to make a blood smear on a cover-glass or slide; the smear is allowed to become air dry, and Wright's stain is poured on at once. The stain dissolved in methyl alcohol also fixes the preparation. The undiluted stain is left on for one minute, then enough distilled water is added to the fluid to cause it to show a dark precipitate, while at the same time the pink of the eosin shows. This dilute stain is left on for two minutes, then the preparation is washed in distilled water for thirty to sixty seconds, finally it is dried with filter paper and examined with an oil-immersion lens.

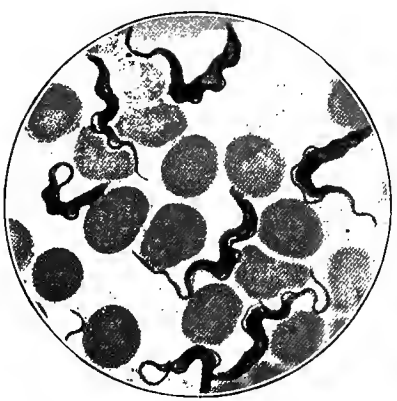
Artificial Culture Media.—The artificial cultivation of trypanosomes was first successfully carried out by Novy and McNeal, whose culture medium consists of ordinary agar distributed to tubes and kept melted at 50° C. Twice the volume of aseptically collected defibrinated rabbit's blood is then added to each tube. When the agar-defibrinated-blood mixture has solidified in a slanting position the condensed water is inoculated with the blood of an infected animal or from a previous culture. This comparatively simple culture medium has enabled Novy and his assistants to obtain much information about the morphology and multiplication of the trypanosomes.

The rat trypanosome, Trypanosoma Lewisii, is very easy to cultivate; the pathogenic trypanosomes are more difficult. Novy, McNeal, and Hare, however, also succeeded in obtaining Trypanosoma Brucei and Trypanosoma Evansii in artificial culture, while Laveran and Mesnil cultivated Trypanosoma Brucei, Trypanosoma dimorphon, and Trypanosoma gambiense.

Novy and Knapp, while working with the flagellates found in the

gut of mosquitoes, could not as easily obtain trypanosomes in pure cultures as when cultivating them directly from the blood in which they are generally the only live microörganisms. They devised the following method, which gave satisfactory results: By means of a glass spatula, made by drawing out the end of a glass rod, a little of the mixed culture of flagellates and bacteria derived from the gut is spread in a series of streaks over six Petri dishes. Ordinary agar may be used in the first three dishes, since the desired dilution is not attained until the last three. The Petri dishes used must be so constructed that they can be sealed tightly with a wide rubber band. The sealed dishes are set aside at room temperature for ten to twelve days. The last two plates frequently contain isolated colonies of flagellates, which can be transplanted in the usual way to the blood agar test-tubes. The flagellates occurring in mosquitoes under ordinary circumstances are, however, not true trypanosoma but the nearly related genera of *crithidia* and *herpetomonas*.

FIG. 189



Trypanosoma Lewisii, non-pathogenic rat trypanosome. × 1000. (Author's preparation.)

FIG. 190

Trypanosoma Evansii, the cause of surra. × 1000. (Author's preparation.)

Pathologic Changes.—It is very probable that pathogenic trypanosomes cause disease by produing or setting free in the blood plasma certain toxins, but practically nothing is known about the latter, and they have not, like certain bacterial toxins and endotoxins, been isolated. The most characteristic pathologic changes of trypanosomiasis are generally a progressive anemia, with disturbances of circulation, congestion, infiltration, and edema, and periodical elevation of temperature, combined, in the later stages, with pareses and paraplegias. The animals finally suffer from rapidly progressing emaciation, which terminates fatally. Apart from the anemic changes of the blood and the bone marrow, and from the presence of the trypanosomes in the blood and other body juices, there are no morbid

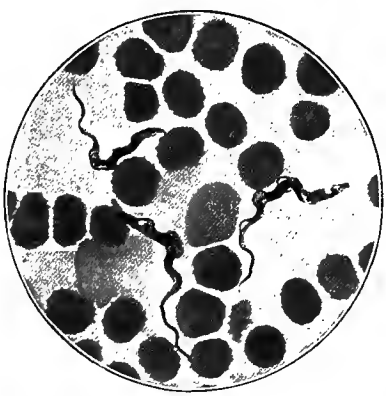
anatomic changes specifically characteristic for trypanosomiases; enlargement of the spleen and the lymphatics, however, is frequently present. When an infected animal is nearing a fatal termination the trypanosomes seen in the blood are usually less mobile and more granular than they are under other conditions.

Diseases Due to Pathogenic Trypanosomes.—The most important diseases of animals and man due to trypanosomes are the following:

Surra.—This disease is due to the Trypanosoma Evansii, and attacks horses and cattle, water buffaloes and carabaos in India, China, the Philippine Islands, and other Asiatic countries.

Nagana.—This disease is due to Trypanosoma Brucei, and is prevalent in Africa, among horses, cattle, camels, wild buffaloes, antelope, wildepests, and probably also elephants. It is invariably fatal in the equidæ and dog, but may terminate in recovery and immunity in cattle. Allied to nagana are a number of other trypanosomiases in various parts of Africa described by Koch (German East Africa), Theiler (Transvaal), Brumpt (Ogaden), Schilling, Ziemann and Martini (Togo), Dutton and Todd (Gambia), and Broden (Congo). Some of these infections are probably identical with nagana, while others are due not to the Trypanosoma Brucei, but to different distinct species. Of the latter the best known is a disease of bovidæ of South Africa described by Theiler under the name of *gall sickness*, or galziekte. It is due to a trypanosome first fully described by Laveran, and named in honor of its discoverer Trypanosoma Theileri.

FIG. 191



Trypanosoma gambiense in human blood, cause of sleeping sickness in man. × 1000. (Author's preparation.)

Caderas, or Mal de Caveras.—This is the trypanosomiasis (*Trypanosoma equinum*) of horses in South Africa, first discovered by Voges.

Dourine.—A somewhat peculiar position among the trypanosomiases is held by dourine, or mal du coit (*Trypanosoma equiperdum*), of the equidæ, a disease transmitted directly from individual to individual by sexual intercourse. It is the only pathogenic trypanosome infection of domesticated animals occurring in European countries, such as Spain, France, Germany, Switzerland, Austria, Hungary, Turkey, and the Balkan States. The trypanosome of dourine was first seen in 1894 by Rouget, and the presence of these parasites in this affection was later established beyond doubt by the researches of

Schneider and Bufford, Nocard and others. In 1901 the disease was first described by Salmon as having been found in the United States, and in 1907 Rutherford, Higgins, and Watson gave an elaborate and

FIG. 192

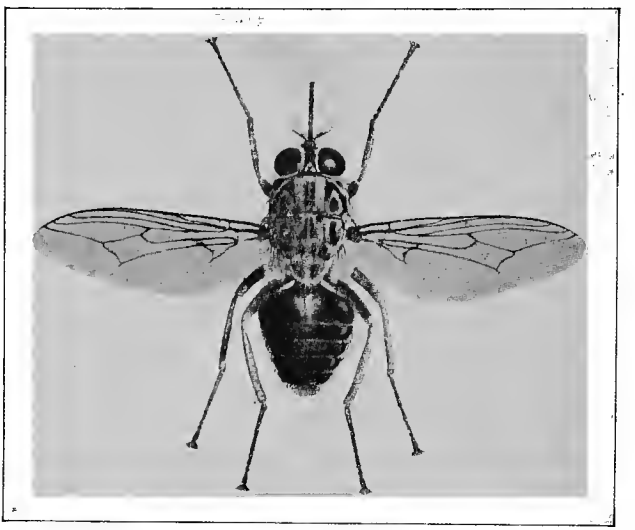
Magnified head of tsetse fly, Glossina morsitans. (Author's preparation.)

lucid account of their observations of, and experiments with, dourine infections encountered in Northwestern Canada.

African Sleeping Sickness.—This is a very fatal disease of human beings, and is due to Trypanosoma gambiense.

Method of Propagation.—Dourine of the horse tribe probably never spreads except through coitus, and in this respect it differs materially from the other known trypanosomiases, which are propagated through biting insects, particularly flies. Nagana is the trypanosomiasis in which the mode of transmission by blood-sucking flies has been most carefully studied by Bruce and others. It is the much discussed *tsetse fly* (Glossina morsitans), which in biting and blood-sucking spreads nagana from infected to non-infected animals. The tsetse fly when at rest can easily be distinguished from other biting but harmless flies. Its wings almost completely overlap like the blades of a pair of shears. In other blood-sucking flies resembling the tsetse,

FIG. 193

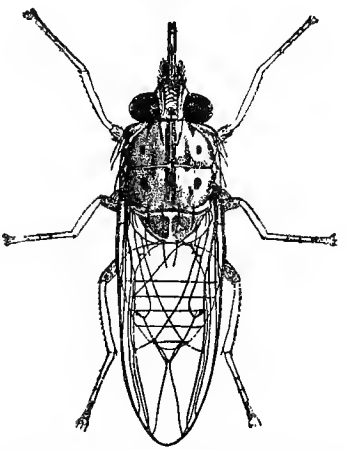


Glossina palpalis, Rob. × 3¾.

the wings when the insects are at rest are always more or less separated. In Africa the tsetse fly is usually found in low-lying, hot, humid regions; it is never seen far from water. Even in the so-called African fly-belt it is not universally found, but is often strictly localized. It bites most furiously during the day, less during the evening, rarely during the night. Both sexes are blood-sucking. They follow the big game in Central and South Africa. It has been found that many wild animals in Africa harbor Trypanosoma Brucei in their blood in small numbers. These animals are not sick, and are evidently in a certain sense immune against the nagana trypanosomes, but the latter when spread by the tsetse fly to domestic animals produce the disease in its virulent form. It has already been said that cattle may

recover from nagana. Whether such animals which have recovered ever become entirely free from trypanosomes or whether they retain them in small numbers without detriment to themselves is a different question. The author during his stay in the Philippine Islands repeatedly examined microscopically the blood of a Government herd of about forty carabaos. These animals had gone through an attack of surra, had apparently recovered from it, were in good flesh, and strong and able to work. Examinations were frequently negative. From time to time, however, a few trypanosomes were found in the blood, and undoubtedly non-immune animals may be infected from apparent immunes harboring such trypanosomes in their blood, without detriment to themselves. This is an important matter from the standpoint of prophylaxis.

FIG. 194



A tsetse fly (Glossina longipennis, Corti, from Somaliland) in resting attitude, showing position of wings. × 3½.

Tsetse flies infected with trypanosomes are dangerous for a short time only. It has been shown by Bruce and others that these insects after having fed on an infected animal can only spread the disease within forty-eight hours. Carnivora may also contract nagana by devouring the flesh and blood of infected animals. When carnivora become infected in this manner it is very probable that this is brought about through injuries in the buccal mucous membrane. Musgrave and Clegg, who experimented with horses, dogs, goats, rabbits, guinea-pigs, monkeys, and cats, have shown in numerous experiments that feeding trypanosoma-infected blood or other material only lead to an infection in the presence of an injury to the mucosa of the gastro-intestinal tract.

The question presents itself whether trypanosomes in the body of their intermediary host (the tsetse fly) undergo a cycle of life changes

resembling that of the hemosporidia of malaria in the body of the anopheles mosquito. Gray, Tulloch, and Koch have claimed that ingested mammalian trypanosomes (Trypanosoma gambiense and Brucei) undergo such developmental changes in the tsetse fly, but Novy has shown that what these investigators believed to be developmental male and female sexual forms were in reality a different species of trypanosoma parasitic in flies and mosquitoes and in no way connected with the pathogenic trypanosoma of nagana. Neither surra nor nagana, the two most important trypanosomiases spread by biting flies, have even been found in man. There is, however, one human infection, the celebrated *African sleeping sickness*, due to the Trypanosoma gambiense, and mentioned briefly above, which is spread by biting flies, and occurs over a large territory. It was first described over a hundred years ago by Winterbottom, and its cause, a trypanosome, was discovered by Dutton in 1901. The Trypanosoma gambiense of human sleeping sickness transmitted by Glossina palpalis is pathogenic for a large number of animals, such as the monkey, lemur, dog, jackal, cat, rabbit, guinea-pig, etc.

Trypanosoma Americanum, n. sp.—Crawley has recently reported that he has obtained from cultures prepared from the blood of normal cattle a new hitherto undescribed species of trypanosoma, which, however, is not present in the blood of the animals as such, but in an unknown form, which only develops into typical trypanosomes in the artificial cultures. Crawley has named this non-pathogenic flagellate, *Trypanosoma Americanum* (novum species). According to the observer the organism can be demonstrated as follows: Blood is drawn under all aseptic precautions from the jugular vein of a cow by means of a sterile syringe and transferred to flasks of 100 c.c. capacity. About 30 to 50 c.c. of blood is taken in each case. In each flask are placed six to eight common faceted beads of rough glass such as are used for cheap necklaces. The flasks containing the blood and the beads are then shaken for a few minutes, which causes the fibrin to collect around the beads. The defibrinated blood is distributed to flasks and tubes containing nutrient (beef) bouillon. Mutton bouillon may also be used. The inoculated flasks and tubes are kept at room temperature. After two or three days trypanosomes appear, and after a few more days they can be seen without the aid of the microscope as little colonies on the surface of the column of blood cells. Those colonies show as small, white plates, and may be 3 to 4 mm. in diameter. They are readily distinguishable from bacterial contamination in that they are flat and sharply circumscribed, while the masses of bacteria are always more or less diffuse and tend to cloud the bouillon. These trypanosomes are not present as such in the freshly drawn blood of cattle, but the evidence is that they develop in the cultures from round or oval bodies. From these lenticular bodies are first formed, then a flagellum is developed, and the flagellate now formed is of the

type of crithidia, these elongate, become trypanosome-like and later are endowed with an undulating membrane. After the typical trypanosome shape has been developed a trophonucleus is seen as a fair-sized vesicle, containing coarse chromatin granules. The kinetonucleus (micronucleus) is usually elongated, forming a long ellipsoid, and stains an almost black-garnet color. The two nuclei are always close together. The undulating membrane is well developed. The parasites appear under two forms, a band-shape and a club-shape. The band-shaped forms are more typical trypanosomes, and are more numerous than the others. In all the cultures the trypanosomes tend to occur in great clusters. These must not be confounded with agglutination rosettes, which present a wholly different appearance. In the clusters nothing in the way of a definite orientation can be made out, while in the agglutinations seen the arrangement was radial.

Observations similar to those made by Crawley had previously been made on Japanese cattle by Miyajima, who, however, believed that the trypanosome which he obtained in cultures represented a stage in the life cycle of a non-pathogenic *piroplasma*. The observations of Miyajima and Crawley as to the development of trypanosomes from healthy cattle when their blood is mixed with nutrient bouillon need further confirmation before they can be fully accepted. It is not to be forgotten that the blood platelets when blood is mixed with certain culture media often assume shapes which may be easily mistaken for trypanosomes.[1]

Trypanosomes in Birds.—Trypanosomes in birds have been studied extensively by Novy and MacNeal. They undertook these studies particularly on account of Schaudinn's claim that trypanosomes in birds were a stage in the life history of the avian intracorpuscular parasite halteridium (Hæmoproteus noctuæ). Schaudinn, on allowing the common mosquito (Culex pipiens) to feed upon the blood of owls infected with halteridium, found in the intestines of about 10 per cent. of these insects large numbers of trypanosomes which he considered a cycle in the life stage of the Hæmocytozoön halteridium. Novy and MacNeal cultivated such trypanosomes from mosquitos in test-tubes on the blood-agar medium. The trypanosomes multiplied readily, but the hematozoa died out. When birds were infected with such pure cultures of trypanosomes no intracellular parasites developed. The cultivation of the bird trypanosomes, according to Novy and MacNeal, is as easy as that of the rat trypanosome. They concluded from their studies that trypanosome infection is very common and widespread in birds, that different avian species harbor different trypanosomes, and that one bird may be infected by several species of trypanosomes. The latter are not pathogenic for birds, but evidently harmless parasites.

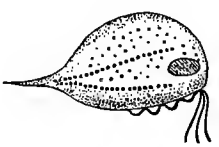
[1] Swingle has recently called attention to this fact in a paper published in the Journal of Infectious Diseases.

CERCOMONAS—TRICHOMONAS—HERPETOMONAS.

While trypanosomes are the most important flagellate from a medical and veterinary standpoint, there are a few others which are parasites of vertebrate animals, though most of them are not pathogenic.

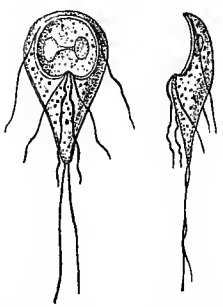
Cercomonas.—These are flagellates of a round or oval body, with a pointed posterior end. The flagella are generally long. The vesicular nucleus is situated near one or two contractile vacuoles. Cercomonas hominis, first described by Davaine, 1854, has been found in the human intestines, urine, and the sputum in disease (gangrene) of the lung. The organism is pear-shaped, with a pointed posterior end, from 10 to 12 micra long; the flagellum is twice as long as the main body. The nucleus is difficult to see. The organism is most frequently found in the intestines of man in chronic diarrheas.

FIG. 195



Trichomonas vaginalis. (Blochmann.)

FIG. 196



Lamblia intestinalis. (Schewiakoff.)

It is, however, not the cause of the pathologic condition, but only finds in the fluid contents of the large intestines conditions favorable to its parasitic existence. The following organisms of this family have been found in domestic and other animals: Cercomonas anatis (duck), Cercomonas canis (dog), Cercomonas gallinarum (chicken). Several species of cercomonas have been found in the intestines of guinea-pigs; two of them, Cercomonas pisiformis and Cercomonas globulus, are believed to be pathogenic for this animal.

Trichomonas.—*Trichomonas vaginalis.*—This organism is found in the vaginal secretion and in the urine of females, occasionally also in the urethra and urine of males. The organism is either pear-shaped or circular. It varies much in size, and the measurements given are from 10 to 25 micra; generally in length from 15 to 25 micra, and the width from 7 to 12 micra. The protoplasm is finely granular and of a greenish hue. The anterior end of the cell carries three, sometimes four, flagella, which are about 10 micra long.

Trichomonas Hominis, or Intestinalis.—This is generally smaller than the preceding one. It is a parasite of the gastro-intestinal

tract of man. It is particularly found in chronic diarrheas, but it is not pathogenic. Prowazek found a similar trichomonas in monkeys and other animals.

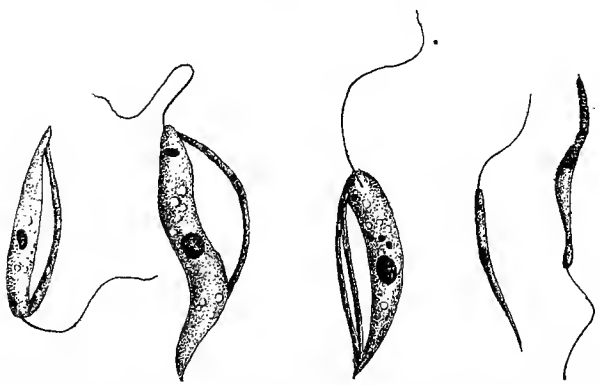
Lamblia Intestinalis.—This is another organism of the order polymastigina, and is found in the intestines of mice, rats, dogs, cats, sheep, and rabbits, and occasionally of man. The organism is beet-shaped and bilaterally symmetrical. It is 10 to 21 micra long, 5 to 12 micra wide; the flagella measure from 9 to 14 micra. At the anterior end the organism possesses a kind of sucking concavity, the margins of which project and appear to be contractile. It has four pairs of flagella, arranged one pair as anterior, one pair as posterior, and two pairs as lateral flagella. The posterior portion of the body forms a tail 2 to 2½ micra long, from which the posterior flagella project outward. The nucleus is bilaterally symmetrical, and each half is oval in shape, each side generally contains a deeper staining nucleolus-like body. The protoplasm is densely hyaline and surrounded by a kind of external pellicle. Contractile vacuoles are not present. The exact mode of division is unknown, but cysts surrounded by a chitinous membrane and possessing four nuclei have been observed. Infection of men and animals is brought about by ingestion of the cysts. Grassi has demonstrated this mode of infection by swallowing cysts. Lamblia in the intestines fastens itself by the sucking apparatus at its anterior end to the intestinal epithelia. The organism, however, does not appear to be pathogenic. Parts of the intestines where numerous lamblia are attached do not show any pathologic changes.

L. Pfeiffer found numerous trichomonas-like protozoa in the intestines of chickens, ducks, and other birds suffering from diarrhea with diphtheritic inflammatory changes in the intestines. It has, however, not been conclusively proved that these trichomonas, or lamblia, were the cause of the intestinal pathologic changes and the death of the fowl.

Herpetomonas.—Herpetomonas, which are nearly related to the cercomonas on one side and to the trypanosomes on the other, are described by Kent as free swimming, elongate or vermicular, highly flexible; the posterior extremity, often the most attenuate, but not constituting a distinct caudal appendage; flagellum single, terminal. Herpetomonas are intestinal parasites of flies and other insects. The best-known species is the Herpetomonas muscæ domestica, the intestinal parasite of the common house fly. It has been studied by Prowazek, who, according to Calkins, describes it as follows: "This organism is elongate and somewhat flattened at one end, which gives rise to the single, long, vibratile flagellum. Apart from the nucleus and blepharoblast the inner protoplasm has no characteristic structures, and the nucleus is of the characteristic mastigophora type, with chromatin granules of more or less definite number. The blepharoblast lies between the nucleus and the flagellum, and is frequently of large size, while from it the base of the flagellum takes

its origin." At the base of the flagellum, just outside of the body, is a small basal granule. Reproduction occurs by longitudinal division. The nucleus divides by a primitive process of mitosis, the granules being equally distributed. This nuclear division is preceded by division of the blepharoblast and of the flagellum, which in this case appears to divide throughout its entire length, instead of one being formed as in some trypanosomes by outgrowth from the blepharoblast. Conjugation has been described by Prowazek as taking place between forms which are not sexually differentiated beyond the fact that one appears to be denser and larger than the other. After conjugation a permanent resting cyst is formed by the fertilized cell, and in this condition the parasite passes from the intestine with the feces of the host. Infection of new hosts usually takes place by ingestion of these permanent cysts with the food.

FIG. 197



Herpetomonas Donovani, unequal division to form slender flagellated individuals. (After Leishman.)

The genus herpetomonas has assumed considerable importance, first, in consequence of Schaudinn's claim that the hemosporidia of birds are a stage in the life cycle of herpetomonas,[1] and secondly, of greater importance, because it is now known that a herpetomonas is the cause of a widespread tropical disease of man.

This disease, which occurs in India, China, Egypt, Arabia, Tunis, Algiers, and other tropical countries, is known as *kala azar*, tropical febrile splenomegaly, dum-dum fever, and by other names. Leishman discovered peculiar bodies in the spleen of a person dead from the disease and his observation was first confirmed by Donovan and later by others, including Marchand, Rodgers, and Christopher. These bodies, first known as the *Leishman-Donovan bodies*, are found in great numbers in the large cells of the spleen or liver. They are round or oval, or rather cockle-shell-shaped, and have two chro-

[1] This, as already explained, is denied by Novy and his associates.

matin masses which are called the macro- and the micronucleus. Rodgers, in 1904, succeeded in cultivating these bodies in blood from the spleen mixed with normal salt solution and neutralized or made faintly acid by the addition of sodium citrate solution. In such artificial cultures the Leishman-Donovan bodies developed into flagellates of the type of herpetomonas, and they have been called *Herpetomonas Donovani* by Laveran and Mesnil. It was also subsequently ascertained that the bed-bug (Cimex rotundatus), in whose intestines the Leishman-Donovan bodies develop into the flagellate type, transmit the disease from the sick to the healthy. Wright found similar intracorpuscular bodies in the granulation tissue of a tropical ulcer. But these have only been studied in sections, and have not yet been cultivated or observed in a flagellate stage.

QUESTIONS.

1. What is the position of trypanosomes in the phylum protozoa?
2. What does the word mastigophora mean?
3. Give a general definition of trypanosomes.
4. Describe their morphology.
5. Define the terms: trophonucleus, kinetonucleus, undulating membrane.
6. Describe the origin and the three parts of the flagellum of typical trypanosomes.
7. Where are trypanosomes found? Are they all pathogenic?
8. Are they intra- or extracorpuscular blood parasites?
9. Describe in detail the method to examine trypanosomes in the live state.
10. Likewise the method to examine stained specimens.
11. In what trypanosomiases are the parasites not found in the blood but elsewhere? Where are they found in these diseases?
12. Describe method of obtaining trypanosomes in pure culture.
13. Which vertebrate trypanosome can be easiest cultivated?
14. What is the method of obtaining pure cultures of flagellates from the guts of mosquitoes?
15. Who discovered the first pathogenic trypanosome; in what disease was it found, and where?
16. What are the most important pathologic changes due to infection by pathogenic trypanosomes?
17. What organism causes surra? What animals are susceptible to surra?
18. What disease is caused by Trypanosoma Brucei; where found, what animals are susceptible?
19. What is dourine? What causes this disease? How is it transmitted?
20. Discuss the general method of transmission of trypanosomiases.
21. Discuss immunity in trypanosomiases.
22. Describe the life cycle of trypanosomes in the body of the tsetse fly.
23. What disease is caused by Trypanosoma gambiense?
24. What is Trypanosoma Americanum, n. sp.?
25. Describe its morphology in artificial cultures and in the circulating blood of cattle.
26. Are birds frequently infected with trypanosomes? What diseases do the latter cause in domestic and other birds?
27. What is the claim of Schaudinn as to the life cycle of bird hemosporidia after their entrance into the gut of the mosquito? Discuss this claim.
28. What is cercomonas? Describe its morphology and its parasitic properties.
29. Describe the morphology and parasitic properties of Trichomonas vaginalis and intestinalis.
30. Describe the morphology and parasitic properties of Lamblia intestinalis.
31. Discuss the position in classification and the morphology of herpetomonas.
32. Where are herpetomonas generally found as parasites?
33. Are any of the herpetomonas pathogenic, if so, describe this pathogenic organism and name the disease it causes.

CHAPTER LIII.

PATHOGENIC SPOROZOA—COCCIDIA—HEMOSPORIDIA—MICROSPORIDIA—SARCOSPORIDIA.

CALKINS defines the subphylum sporozoa as parasitic protozoa without motile organs, but capable of moving from place to place by structural modifications of one kind or other; reproduction, either simple or multiple, but mainly by spore formation, which is either asexual (schizogony) or sexual (sporogony). This subphylom is very rich in genera divided into a number of classes and orders. Only four orders, however, come within the scope of this work, namely:

A. *Coccidia.*—These are cell-infecting protozoa, which usually reproduce by schizogony and sporogony, thus giving a life cycle with an alternation of asexual and sexual generations. After fertilization the oösphore forms sporoblasts (mother cells giving rise to spores), which may or may not (asporocystea) be covered by a sporocyst membrane, and which may each become transformed into one or several young reproductive spores (sporozoites).

B. *Hemosporidia.*—These are intracorpuscular blood parasites which may change from a permanent to an intermediate host, or which may be confined to one host.

C. *Microsporidia.*—The young vegetative cells are more or less ameboid; the spores are very minute, pyriform, with only one capsule, which is invisible in the fresh state. They are intracellular parasites of invertebrates.

D. *Sarcosporidia.*—These are sporozoa in which the initial stage is passed in muscle cells of vertebrates.

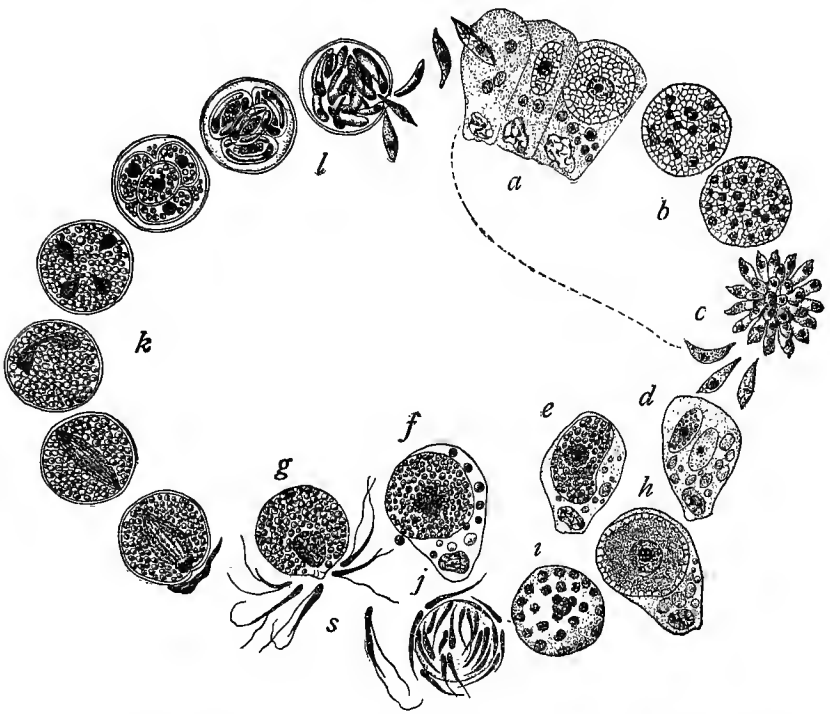
COCCIDIA.

Coccidia are all cell parasites; they are found in vertebrate and also in lower animals, such as mollusks and insects. They are parasitic in the cells of the gastro-intestinal and genito-urinary tract. They are generally round or oval. The protoplasm does not show a differentiation into endoplasm and ectoplasm. As a rule, it contains granules, which differ in staining affinities toward various stains. The nucleus is usually situated in the centre, is vesicular, and contains in its interior a granule, which has received the name karyosome. Propagation occurs alternately in sexual and then asexual manner, so that a definite, complicated cycle of life is formed. The spores

(merozoites) developed after asexual schizogony spread the infection within the same host (auto-infection), while the sporozoites formed after sexual reproduction may spread the infection to a new host.

The life cycle of a coccidium may, therefore, be outlined as follows (Doflein, Schaudinn): A cyst containing sporozoites, the product of sexual propagation, is taken into the gastro-intestinal tract of an animal.

FIG. 198

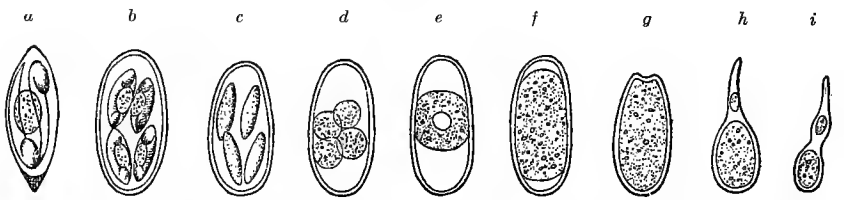


Life cycle of Coccidium schubergi. (After Schaudinn.) Sporozoites penetrate epithelial cells and grow into adult intracellular parasites (*a*). When mature the nucleus divides repeatedly (*b*), and each of its subdivisions becomes the nucleus of a merozoite (*c*). These enter new epithelial cells, and the cycle is repeated many times. After five or six days of incubation the merozoites develop into sexually differentiated gametes; some are large and well stored with yolk material (*d*, *e*, *f*); others have nuclei which fragment into many smaller particles ("Chromidia"), each granule becoming the nucleus of a microgamete, or male cell (*d*), *h*, *i*, *j*). The macrogamete is fertilized by one microgamete (*g*), and the copula immediately secretes a fertilization membrane, which hardens into a cyst. The cleavage nucleus divides twice, and each of the four daughter nuclei forms a sporoblast (*k*) in which two sporozoites are produced (*l*).

The cyst wall or membrane is dissolved, the sporozoites become free, and a number of them enter epithelial cells, penetrate into their nuclei, and grow into forms, which in some coccidia already show a differentiation into cells that will later furnish the male and others that will furnish the female gametes. Both kinds of cells then divide asexually and form male and female gametes, respectively. These

may invade new epithelial cells of the same host, and may again subdivide in an asexual manner. After this has occurred a number of times the female merozoites begin to grow and to accumulate nutritive material in their protoplasm. They then fall out of the cell which they have infected and destroyed, and they reduce the amount of their nuclear chromatin by the formation of polar bodies and the expulsion of some chromatin. These are the phenomena of maturation through which a germ cell must always pass before it can be fertilized by another germ cell, which likewise has had to go through the same process of maturation. While the female merozoites have in this manner become the *macrogametes*, the male merozoites have gone through a series of nuclear divisions and have formed a number of spindle-shaped flagellated *microgametes*. The latter penetrate into the macrogametes, and this, of course, constitutes the act of fertilization. The *copula* formed by the union of the microgametes and macrogametes later divides into two sporoblasts, or mother cells, giving rise to the sporozoites, which are the result of the sexual fertilization and propagation which have occurred. The *oöcyst* containing the spores may again be taken up by a new host, and the cycle can begin anew.

FIG. 199



Showing spore formation in *Coccidium cuniculi*, from the liver of a rabbit: *a* and *b*, young stage in the epithelial cells of the gall-ducts (the small oval is the cell nucleus); *c*, *d*, and *e*, the fertilized oöcyst; in *d* the protoplasm is beginning to shrink away from the cyst wall, and in *e* it has contracted into a spherical form; *f*, segmentation into four sporoblasts; *g*, elongation of the sporoblasts to form spores; *h*, four complete spores in the oöcyst; *i*, single spore more highly magnified, showing the two sporozoites and a small quantity of residual protoplasm. The life cycle has been fully worked out by Simon. (After Balbiani, from Doflein.)

Coccidium Cuniculi.—The best and probably earliest known coccidium pathogenic to mammals is the Coccidium cuniculi, or oviforme, first described as Psorospermium cuniculi by Rivolta (1878). It is parasitic in the intestinal epithelium and the liver cells of wild and tame rabbits, and it sometimes causes fatal epidemics among rabbits of laboratories and places where they are bred. The spore cysts are taken up with food soiled by feces from animals harboring the infection. After the cyst membrane has become dissolved the sickle-shaped spores penetrate into the interior of the intestinal epithelial cells of the host. The asexual forms are 20 to 50 micra long and 20 to 35 micra wide; 30 to 200 merozoites are formed in asexual reproduction. The coccidia disease of rabbits lasts from one to two weeks, and leads to fever, diarrhea, and emaciation, with

a yellowish mucopurulent discharge from the mouth and nose. The liver is very much enlarged, and shows on section densely crowded. grayish-white nodules from the size of a millet seed to a hazelnut, These nodules are surrounded by a capsule, and often contain a smeary mass composed of degenerated liver epithelia, leukocytes, and the pathogenic coccidia. The invasion occurs from the bile-ducts, the epithelial cells of which, while being destroyed in some places, proliferate in other places as a result of the inflammatory stimulus. Rabbits which recover from the infection contain the oöcysts for a long time in their appendix and their gall-bladder. Cicatrix formation and calcification in the liver are often seen after coccidiosis in rabbits.

Another coccidium parasitic in the intestines of rabbits has been named Coccidium perforans by Leuckart.

Coccidiosis in Cattle and Other Animals.—There is a disease of cattle known as coccidiosis intestinalis, dysenteria coccidiosa bovum, "Rothe Ruhr der Rinder" (German), "flux de sang" (French), characterized by bloody discharges from the bowels, without fever, but with progressive emaciation in severe cases. The disease occurs particularly among young animals and on marshy pastures. Zschokke, Guillebeau and Hess, and Degoix have shown a coccidium which is 18 to 25 micra long, 13 micra wide, in the stools of sick animals and in the gastro-intestinal tract. According to Guillebeau these coccidia form four spores at a temperature of 20° to 30° C., but at 39° C. numerous small round spores of 4 to 7 micra diameter. Young cattle can be infected artificially with these coccidia by feeding them, and after an incubation of three weeks they develop typical attacks of the disease. The coccidia have been found in the intestinal epithelia, particularly in those of the crypts of Lieberkühn. The gastric mucosa in this coccidial infection shows inflammatory and hemorrhagic changes. In fatal cases all the organs show the signs of anemia and cachexia.

Coccidiosis in sheep has been reported by Rivolta, Leuckart, Nocard, Cooper, Curtice, Stiles, McFadyean, and others. The symptoms are similar to those of coccidiosis in cattle; the coccidia are found in the intestinal epithelia, but they have not been found in the feces.

Coccidium tenellum, claimed to be the cause of white diarrhea in chickens, has been mentioned in Chapter XXIV under the head of Bacterium Pullorum.

Coccidiosis renalis is the name given to a condition in which after death due to progressive cachexia, coccidia have been found in the kidneys. Ralliet and Lucet have reported such cases in geese, and Paechinger has reported one case in a horse and one in a dog.

A skin disease of hogs known as hypotrichosis localis cystica, spiradenitis coccidiosa, "Schrotausschlag der Schweine" (German), characterized by a chronic eruption of the skin, is claimed to be due to the Coccidium fuscum of Alt, which invades the epithelia of the sebaceous glands.

HEMOSPORIDIA.

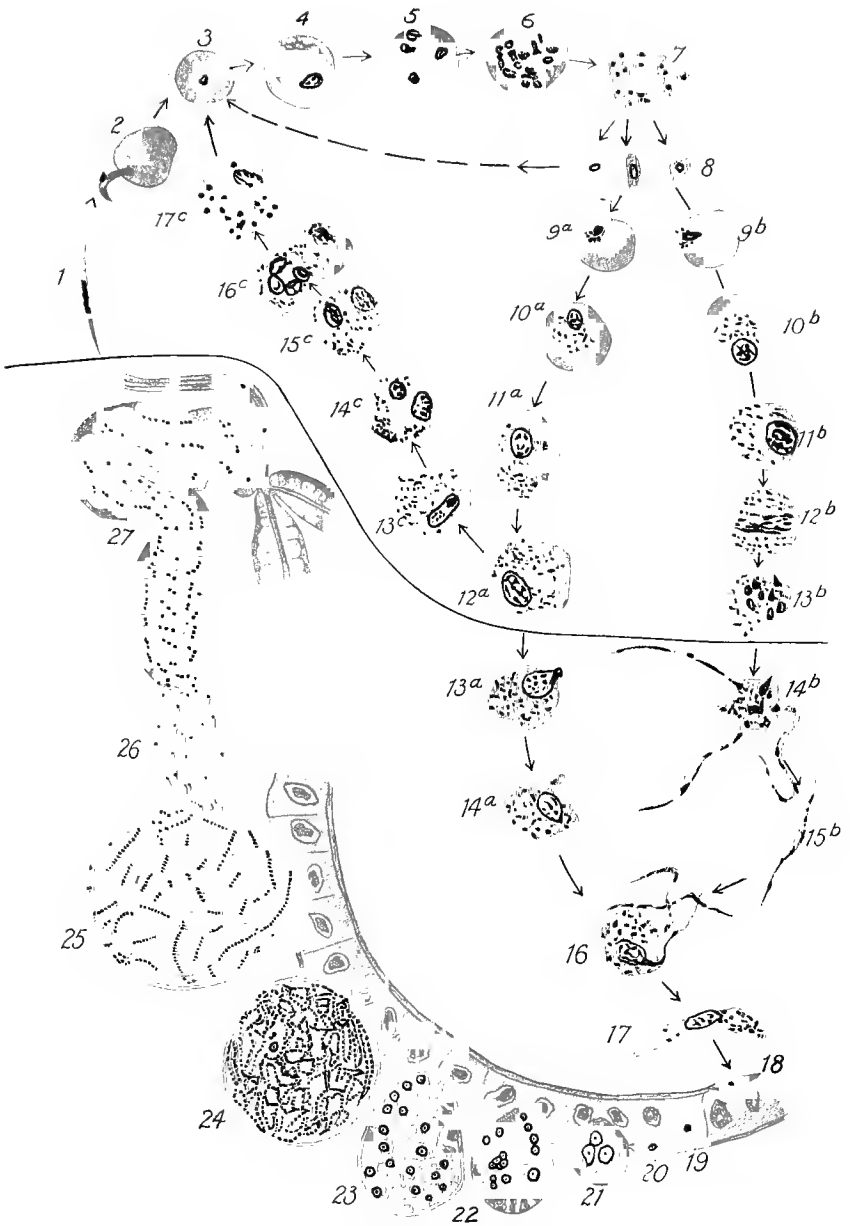
The hemosporidia include some of the most important pathogenic protozoa, causing disease in man and domestic animals, namely, the plasmodium which causes malaria in man and monkeys; hemoproteus, the cause of malaria in birds; piroplasma, or Babesia, the cause of piroplasmoses, or hemoglobinurias, in several species of domestic animals. To the piroplasmoses also belongs Texas fever of cattle. This group of diseases will be considered separately in the next chapter.

Malaria.—Probably no other disease is so widely prevalent among human beings throughout the world, except in the most northern and southern latitudes, as malaria. It is spread from the infected to the non-infected by mosquitoes of the genus anopheles.

Malarial parasites were first seen in the blood of patients and considered to be the cause of the disease by Laveran in 1880. Marciafava and Celli in 1885 gave a more detailed description of the microörganisms, and proposed for them the name of Plasmodium malariæ, under the erroneous impression that they were dealing with a vegetable microörganism. This name is still retained, though the malarial organisms are now properly classified among the subphylum sporozoa, order hemosporidia. Schaudinn divides the species into three varieties, namely, *Plasmodium vivax* (the parasite of tertian malaria), *Plasmodium malariæ* (the cause of quartan malaria), and *Plasmodium falciparum* (the organism of quotidian or estivo-autumnal malaria).

The malarial fevers are characterized by a very definite recurrence of elevations of temperature after different periods of time. The fever curve may rise daily, and reach its maximum at an almost constant time of each day; this is the so-called *quotidian* type. Or the apex of the fever curve, generally ushered in by a chill, may occur and re-occur after forty-eight hours; this is the *tertian* type. Or it may occur always after seventy-two hours, this is the *quartan* type. These febrile and afebrile periods depend upon the natural life history of different varieties of malarial parasites. If a person is infected by the bite of a mosquito which carries the parasites as the intermediate host the plasmodia get into that person's blood and there multiply. For a certain time the number of parasites is comparatively small, no symptoms develop, and the patient is then in the period of incubation. Then an outbreak occurs, characterized by chills, followed by fever. This is repeated after twenty-four, forty-eight, or seventy-two hours, according to the variety of infecting plasmodium. It can be easily shown that these outbreaks always occur shortly after the time when the intracorpuscular parasites break up into merozoites, or asexually produced spores. As soon as these are liberated from the corpuscle which they have destroyed

PLATE XII



Life-cycle of Plasmodium Vivax. (After Grassi and Schaudinn.)

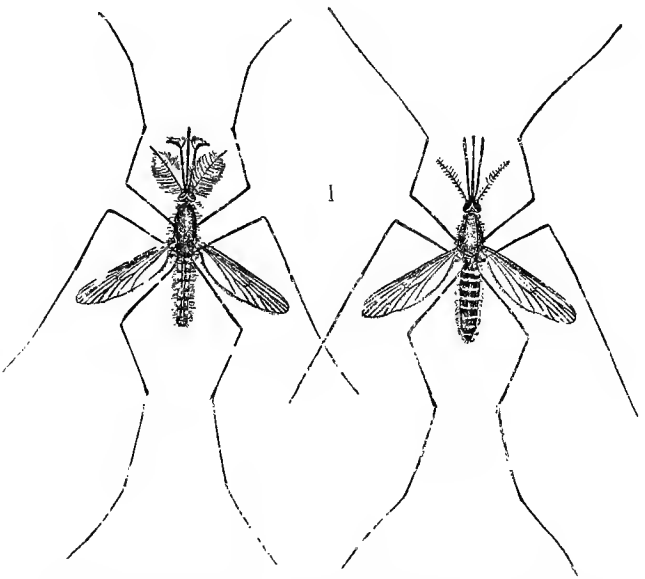
The human cycle is above the transverse line, somewhat rearranged by Kisskalt and Hartmann. The cycle in the mosquito is beneath. 1 to 7, schizogony; 1, sporozoite; 2, entrance of the sporozoite; 3 and 4, growth of the schizont; 5 and 6, nuclear division of the schizont; 7, formation of the merozoites; 8, merozoites; 9*a* to 12*a*, growth of the macrogametocyte; 9*b* to 12*b*, growth of the microgametocyte; 13*c* to 17*c*, parthenogenesis of the macrogametocyte; 13*a* and 14*a*, maturation of the macrogamete; 13*b* and 14*b*, growth of the microgamete; 15*b*, microgamete; 16, fructification; 17, oökinet; 18 to 20, entrance of the oökinet into the stomach wall of the mosquito; 20 to 25, sporogony; 22 and 23, nuclear multiplication in the sporont; 24 and 25, formation of the sporozoites; 26, passage of the sporozoites to the salivary gland; 27, salivary gland of the mosquito with sporozoites. (Magnification, 1 to 17*c*, 1200 to 1; 18 to 27*c*, 600 to 1.)

they invade fresh corpuscles, and either directly or indirectly are responsible for the chill, fever, prostration, etc.

The parasites of malaria have a double cycle of reproduction. Asexual reproduction (Schizogony) by spore formation (merozoites) occurs in the blood of man or monkeys; the sexual cycle of reproduction by copula formation of sexually differentiated gametes occurs in the body of the intermediate host, the mosquito of the genus anopheles.

Plasmodium Vivax.—This is the cause of tertian malarial fever. After its entrance into the body of man it is first seen in the interior of red blood corpuscles as a small, quite motile, hyaline body, variable

FIG. 200



Anopheles maculipennis: adult male at left, female at right. (Howard.)

in shape on account of the contractility and ameboid motion of its protoplasm. After a time the latter contains reddish-brown, rather fine, diffusely distributed pigment, which increases in amount. While the perfectly hyaline bodies are difficult to see under the microscope the parasites are easily visible after they have formed pigment, particularly as the granules in fresh blood are in constant motion in consequence of protoplasmic contractility and currents. About forty-eight hours after its first entrance into the red blood corpuscle the plasmodium has reached a large size, and now fills the enlarged erythrocyte almost completely. The parasite now has lost its ameboid motion, is round in shape, and divides by segmentation into twelve to twenty-four merozoites (asexually produced spores).

The merozoites, which are from 1 to 3 micra and more in diameter, get into the blood plasma and from there infect new erythrocytes. The early differentiation of gametocytes occurs in man, but the completion of this change only takes place after mosquitoes have taken up the parasite with the blood. The gametocytes then become fully developed, form gametes, and these go through a process of maturation in the intestinal tract of anopheles. Microgametes then fertilize the macrogametes, and the copula formed has been called *oökinet* by Schaudinn. This oökinet penetrates into the submucosa of the gut of the mosquito, and grows considerably in size. After several days its nucleus divides, the cytoplasm likewise segments, and the naked sporozoites are then formed. These circulate in the body of the mosquito, and many of them finally get into the salivary glands and from there into the proboscis of the biting insect, through which they are ultimately discharged into the body of man. Then the asexual cycle starts anew until merozoites are again taken up by anopheles, in which they pass through the sexual part of the cycle.

Plasmodium Malariæ.—The parasite causing the quartan type of malaria is like the preceding one first seen as a hyaline body in the red blood corpuscle. It is, however, not as lively motile as the plasmodium vivax. The pigment granules which subsequently form are larger than in the case of the tertiary parasite, and are arranged in a regular peripheral and not in a diffuse manner. The whole parasite remains smaller and the infected red blood corpuscle does not become abnormally large, is not very pale, but rather of a dark greenish color. At the end of the third day the Plasmodium malariæ is full grown, and is much more highly refractive than the Plasmodium vivax. From eight to twelve merozoites are then formed, arranged in a regular rosette. The merozoites after being set free in the blood plasma invade new corpuscles. The sexual part of the cycle in the mosquito is like that of the preceding variety. When blood containing either the Plasmodium vivax or Plasmodium malariæ is obtained by the prick of a needle and allowed to fall on a slide, covered with a cover-glass, protected against evaporation, and watched under the microscope, the formation of flagellated forms can be observed. Fully grown plasmodia filled with very actively motile pigment form wavy, slender prolongations of the protoplasm, several times as long as the diameter of the main body of the parasite. The *flagella* exhibit a very lively whip-like motion. The cells which have undergone this change are the microgametocytes, which produce the microgametes by the breaking loose of the flagella after they have been provided with nuclear substance from the mother cell which formed them. While the formation of the flagellated organisms occurs, round parasites, with pigment collected in larger masses in a peripheral manner, can be seen. These plasmodia are the macrogametocytes. These sexual forms, which frequently can be seen under the microscope in drawn

blood, are always formed in the gut of the mosquito as the first step in sexual reproduction as outlined above.

Plasmodium Immaculatum or Falciparum.—According to Marchiafava and Bignani and Craig this occurs in two varieties, namely, the quotidan and the tertian. These are described by Craig as follows: The *quotidian parasite* after invading the red blood corpuscles is first indistinct, but later becomes clear-cut and refractive. There are round forms, but the most common form is the ring form. While most observers think that the ring form is only apparent, due to a very thin centre, Craig believes that this type of plasmodium generally forms real rings in the interior of the erythrocytes. Schaudinn believes that the ring form is due to a large vacuole in the centre of the organism. The ameboid motion of the rings is very active; the infected red blood corpuscles are frequently undersized, and they may be crenated. One corpuscle may contain two or three rings. The Plasmodium falciparum assumes the shape which has been likened to a *signet ring*. This form is brought about by a collection of most of the protoplasm in one point, while the remainder is arranged as a thin circular strip. The pigment first appears in the thickest portion of the signet ring. These formations are more common in the tertian than in the quotidian type. The pigment is rather scanty, but very dark in color, and collected somewhere at the edge of the parasite. Sometimes the pigment consists of a very few distinct granules only. Segmenting forms are rarely seen in the peripheral blood, but they are common in the spleen, from which they may be obtained by puncture. At the time of segmentation the pigment becomes collected at the centre. There are six to eight very small round or oval merozoites formed. In the circulating blood Plasmodium falciparum forms the *crescents*, which are so characteristic for this variety of malarial parasite. These crescents are curved. Sometimes they fill the greater part of a red blood corpuscle or even protrude out of it at one or both ends, and they may finally show a remnant of the corpuscle as a cap lying in the concavity. The pigment is found in the centre of the crescent, where it is often arranged in a regularly circular manner. The crescents are the macrogametocytes of the plasmodium falciparum, and a number of observers have seen a binary division of the crescents in the infected blood.

The *tertian subvariety* of the Plasmodium falciparum in its early stage after invasion of the blood corpuscles is larger than the quartan subvariety, very highly refractive, and the signet-ring forms are still more marked. Ameboid motion is less rapid. The hyaline forms become pigmented in twenty to twenty-four hours; the abundant pigment consists of very fine reddish-brown granules, which are generally motile. As growth progresses the ameboid motion is lost. Segmentation occurs after forty-eight hours, and the organism then occupies about one-half of the infected cell. From ten to fifteen merozoites are generally formed, sometimes as many as twenty-

four. Segmentation is generally not seen in the peripheral blood, but it can be shown in blood drawn by puncture from the spleen.

Examination of Blood.—Examination of the blood for the plasmodium of malaria is made on unstained fresh and on dried specimens. The latter are best stained with the Romanowski stain or one of its modifications, such as the Wright stain.

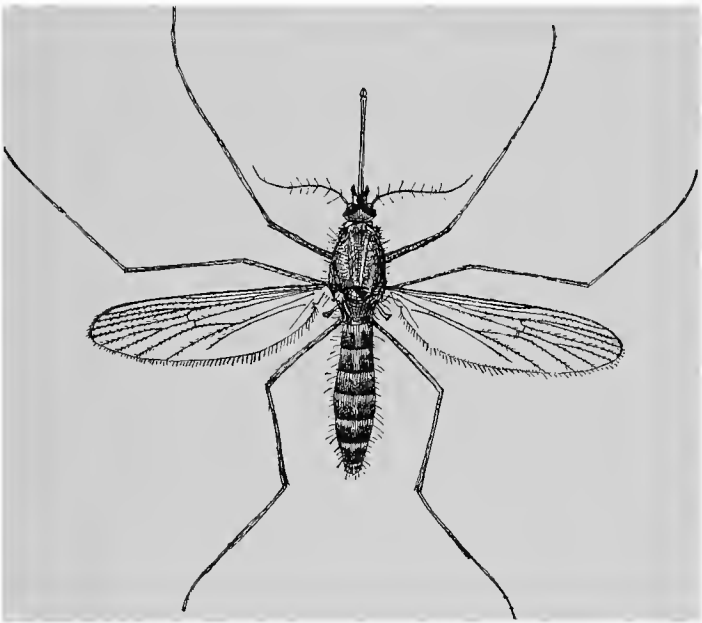
Hemosporidia in Birds.—Birds frequently harbor trypanosomes in their blood, as has been stated previously. Two kinds of hemosporidia have also been found.

Proteosoma, or *Cytosporon danilewsky,* or Hemameba relicta is found in birds of the sparrow family, in predatory birds, pigeons, crows, etc. The life cycle of this hemosporidium is described by Ruge as follows: The youngest parasites are seen in the erythrocytes of birds as a small, round, refractive, sharply defined body, with one minute pigment granule. The young proteosoma is generally situated near one pole of the blood corpuscle, or it may be near its nucleus. The parasite is not motile; it grows rapidly and causes the nucleus of the erythrocyte to move or turn away from it. During growth the pigment increases and becomes lumped together. Afterward the organism breaks up into six to eight merozoites, which are arranged as a rosette or in fan-shape. The largest forms break up into twelve to fifteen spores. The corpuscles infected with the dividing parasites lose their regular shape and burst. The free spores then invade fresh blood corpuscles. Sexually differentiated gametes, however, are also formed in the blood of infected birds. Their further development occurs in the intermediary host, a mosquito (Culex pipiens). The macrogametes become large and round; the flagellated microgametes are formed in a manner resembling the formation which occurs in anopheles in the case of the human malarial plasmodia. The *oökinetes* are formed in the stomach of the mosquito about twelve hours after it has taken up the infected blood. Seven to ten days later the sickle-shaped sporozoites are found in the salivary glands of the insect. Sporozoite development, however, occurs for a short time only, during temperatures of 24° to 30° C.; between 15° to 23° C. their development is much retarded, and at lower temperatures it ceases entirely.

Halteridium, or *hemoproteus,* infects frequently the red blood corpuscles of predatory birds, singing birds, and particularly pigeons. In tropical and subtropical countries, R. Koch found pigeons very generally infected. Halteridium is generally seen in its typical dumb-bell shape in close apposition with the nucleus of the erythrocyte. The dumb-bell-shaped parasites, according to Ruge, occur in birds in two types, a hyaline form representing the male, and a finely granular form representing the female element. MacCallum has observed how the microgametocytes of the halteridium become flagellated and how the microgametes penetrate into the macrogametes. The life cycle of halteridium, however, is not yet completely known.

Schaudinn has claimed, as previously stated, that Hemoproteus noctuæ (the parasite of the owl) develops in the body of Culex pipiens into a flagellate, a trypanosome, or allied organism; but Novy and his co-workers maintain that the flagellates seen by Schaudinn in culex are not a phase in the life cycle of hemoproteus but simply a parasitic flagellate of the insect.

FIG. 201



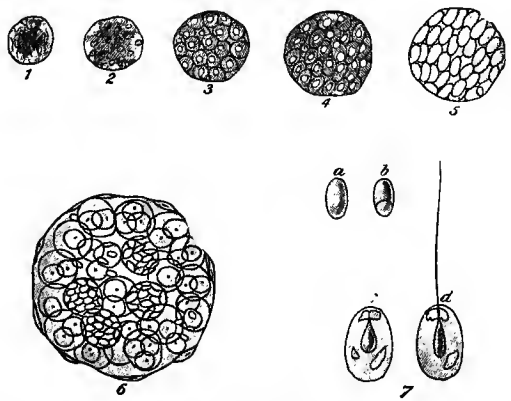
Culex pipiens; adult female. (Howard.)

MICROSPORIDIA.

Nosema Bobycis.—Nosema, or Glugea bombycis, is the most widely known microsporidium. It is the cause of the silkworm disease, pebrine (French), studied by Pasteur. During the years 1854 to 1867 this microörganism is estimated to have caused losses in France amounting to about $200,000,000. The silkworm affection caused by this microsporidium was the first disease studied by Pasteur, and to a large extent conquered by his prophylactic measures. These studies initiated him into the field of preventive medicine, where he later gained such immortal fame. Nosema bombycis, therefore, is a pathogenic organism of great historical interest. It is believed that the caterpillar of the silk moth (Bombyx mori) infects itself with its food with the parasites. The caterpillars, extensively infected, die before they have had an opportunity to form chrysalides inclosed in cocoons of silk. Those less infected can go on to full development

as male and female moths. Since the sexual organs are infected with the parasites they transfer the infection to the ova and from these to the young caterpillars. In this manner the infection may be continued from generation to generation, bringing about both great mortality and an inferior quality of cocoons. Pasteur showed how to distinguish microscopically the infected from the non-infected ova, and in this manner enabled the breeders of silkworms to weed out the disease.

FIG. 202

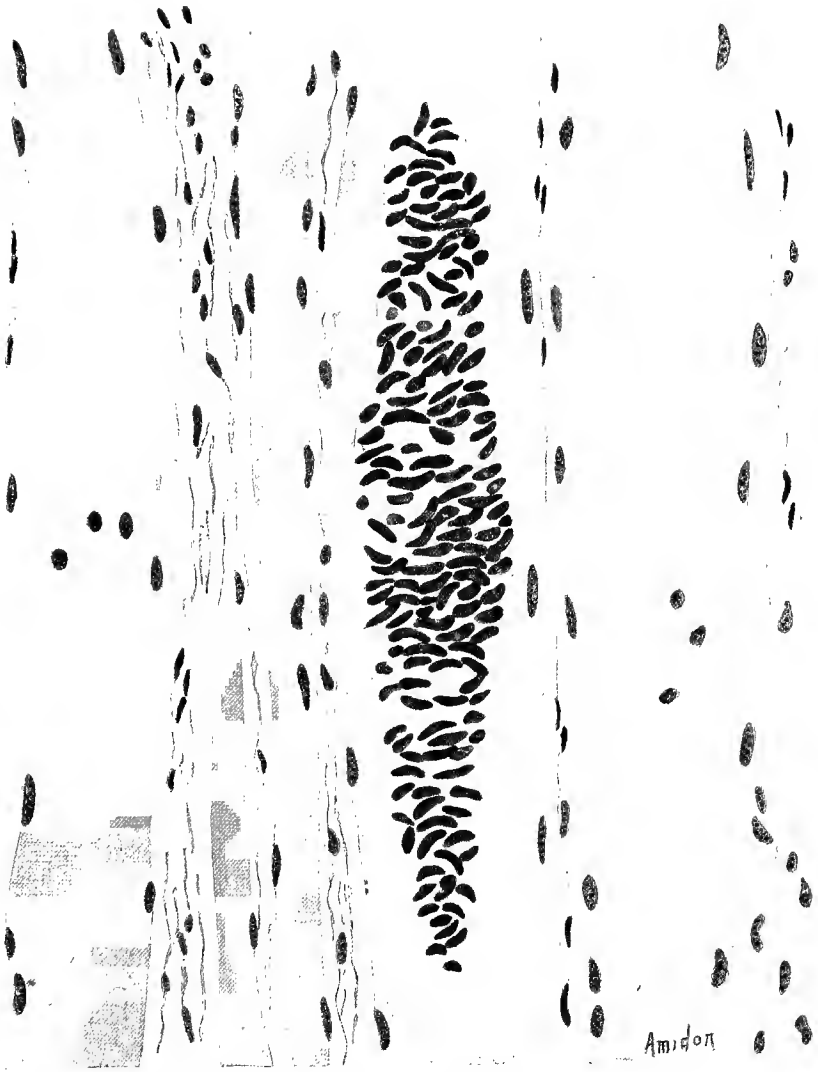


Nosema bombycis: 1 to 5, spore formation; 6, infected follicle of testicle; 7, spores; *a*, *b*, fresh; *c*, *d*, treated with nitric acid. The acid causes them to swell up and increase in size by at least a half, at the same time making the polar capsule distinct. In *d*, the filament is extruded. (After Balbiani.)

SARCOSPORIDIA.

Sarcosporidia are protozoan parasites occurring in the muscle fibers of a large variety of animals, such as hogs, sheep, cattle, horses, dogs, cats, rabbits, rats, mice, monkeys, chickens, and some other domestic and wild birds. They have also been occasionally found in the muscles of man. In the muscles of affected animals sarcosporidia form elongated sausage-like spore sacs, which have been known for a long time as *Miescher's* or *Rainey's tubules*. These spore sacs generally measure from $\frac{1}{2}$ to 4 mm. in length, but there is a sarcosporidium (Balbiana gigantea) found in the esophagus of sheep which may attain the size of a hazelnut. Older spore sacs sometimes show a double membrane, the outer one exhibiting a radial striation, as if it were provided with short, rod-like cilia. The real character of this structure has not yet been clearly made out; it is now more generally believed that the striæ represent fine pore-canaliculi. The interior of the sac is divided into compartments by fine partition walls arising from the inner membrane. Included in these chambers are the *sporoblasts* and their *spores*. In the entoplasm of the smallest sacs, balls 4 to 5 micra in diameter, which show an indistinct nucleus,

PLATE XIII



Sarcosporidia in Muscle of Cattle.

are seen. The latter subsequently divides the cytoplasm segments, and in this manner the balls become sporoblasts and their contents are transformed into numerous crowded, curved, oval, sickle- or crescent-shaped spores. It is not known how the sarcosporidia first gain entrance into animals, nor is their life cycle well known. It is believed that part of it occurs in an intermediate host, and it has been claimed that mollusks (snails) play this role. Sarcosporidium infection in animals is, as a rule, a harmless process. The parasites, however, may be very numerous throughout the entire muscular system, and may so interfere with its nutrition and function; or the sacs may be in locations where they may do harm. The giant sarcosporidia in the esophagus of the sheep, for example, may interfere with deglutition and respiration. A few cases of sarcosporidia infection of the laryngeal muscles of horses have been reported; the author has seen such a case in which the larynx became the seat of considerable inflammatory infiltration, with respiratory disturbances. Sarcosporidia can be studied from fresh material, unstained and also in stained sections of infected muscles.

Sarcocystis miescheriana is the sarcosporidium most commonly found in hogs. The sacs are from 0.5 to 4 mm. long and 3 mm. wide. The pansporoblasts (the balls which subsequently develop the spores in their interior) are 5 to 6 micra in diameter. If pork is extensively infected by these sarcosporidia it is "off color," yellowish or grayish red. The sacs often show the evidences of leukocytic infiltration and of calcareous degeneration.

Sarcocystis bertrami, generally 9 to 12 mm. long, is found in the muscles of the horse. It may cause interstitial myositis, and may become dangerous when located in the muscles of the larynx. It sometimes affects the muscles of the hind leg of young horses and causes lameness.

Sarcocystis tenella infects the muscles of sheep and goats. The organism varies considerably in length, namely, from 40 micra to 2 cm. The sac membrane is very delicate in young parasites, but becomes thick and tough when they grow older. The large cysts contain a layer of sporoblasts along the interior of the membrane, but the centre is composed of an empty meshwork only. The spores are first perfectly round, but small and kidney-shaped after their full development. This sarcosporidium invades many of the muscles of sheep and goats, and also the heart muscle and its endocardium.

Sarcocystis lindemann has been found by R. Koch and by Kartulis in Africa in the muscles of man and also by others a few times in other parts of the world.

Balbiana rileyi, another sarcosporidium, was found by Stiles in the muscles of ducks in the United States.

QUESTIONS

1. What are the most important common characteristics of the protozoan subphylum sporozoa?
2. Give a definition of the order of coccidia.
3. Give a definition of the order of hemosporidia.
4. Give a definition of the order of microsporidia.
5. Give a definition of the order of sarcosporidia.
6. In what kind of animals and in what tissues are coccidia found as pathogenic parasites?
7. Describe the cytoplasm and the nucleus of typical coccidia.
8. Describe the asexual and sexual life cycle of a coccidium.
9. What is meant by polar bodies? What by the process of maturation?
10. Explain the terms macrogametes and microgametes and copula.
11. Describe the most important pathologic changes and the symptoms of coccidiosis in rabbits.
12. What is the other name of Coccidium cuniculi? Describe its morphology.
13. Describe coccidiosis in cattle.
14. What other domestic animals may suffer from coccidiosis?
15. What is hypotrichosis localis cystica of hogs? What causes it?
16. Name some hemosporidia causing important diseases in man and animals.
17. What kind of a disease is malaria? How is it spread from the infected to the non-infected?
18. Describe the Plasmodium vivax in the blood of man.
19. Describe its sexual cycle in the anopheles mosquito.
20. What is an oökinet?
21. Describe the Plasmodium malariæ.
22. Describe the Plasmodium falciparum.
23. What is the cause of the regularity of the fever curve in the various forms of malaria?
24. Name the hemosporidia in birds.
25. What other protozoa infect the blood of birds?
26. Describe proteosoma.
27. Describe halteridium or hemoproteus.
28. Has this hemosporidium a flagellate stage in its life cycle?
29. What kind of an organism is Nosema or Glugea bombycis?
30. Describe the morphology of sarcosporidia and state where they are found. Are they very pathogenic?

CHAPTER LIV.

PIROPLASMA BOVIS—TEXAS FEVER AND PIROPLASMOSES IN OTHER ANIMALS.

PIROPLASMA BOVIS.

Occurrence and Historical.—Texas fever is a disease of cattle due to a protozoan microörganism infecting the blood plasma and the red blood corpuscles, and now generally known as Piroplasma bigeminum, or Babesia bigemina. The disease has been and is known under a variety of names, such as splenic fever (this name, however, is now more commonly used for anthrax), Spanish fever, Mexican fever, Southern cattle fever, Australian tick fever, Tristeza, red water, black water, hemoglobinuria of cattle, paludism of cattle, piroplasmosis of cattle, etc. The disease has undoubtedly existed in the old world, where it was formerly known as wood and moor ill, for a long time. It first attracted attention both in Europe and America about the middle of the last century. At this time the disease was studied by veterinarians in Russia and France, and also became the subject of much inquiry in this country, when cattle coming from Texas introduced the disease into Indiana and Illinois, where its ravages became alarming, and when it likewise appeared in cattle brought from the West to the slaughtering houses of New York. A commission appointed in the latter State studied the disease and issued a report in 1868. It described the symptomatology and pathology of the disease correctly, but did not ascertain its cause. Later investigators accused various bacteria of being the cause of the disease, but erroneously, as was subsequently shown. In 1888 Babes studied the disease in Roumania, and reported that he had discovered in the interior of the red blood corpuscles of animals sick with hemoglobinuria diplococci-like bodies which could be stained with methylene blue, but which could be cultivated only with difficulty. Babes thought that these diplococci-like bodies were neither bacteria nor protozoa, but some organism intermediate between them. While Babes undoubtedly saw and correctly described the organisms causing Texas fever in cattle, he was in error concerning their alleged cultural properties, and had no correct conception of their mode of entrance into the body of the infected animal, which he thought was through the drinking water. The etiology of Texas fever was cleared up completely in 1893 by Theobald Smith and Kilbourne. They saw the infecting protozoa, described them correctly, and showed that the

mode of infecting cattle was by transmission through biting and blood-sucking ticks. Their work was afterward confirmed in other parts of the world in the observation of identical or at least similar blood infections of cattle in Finland, Italy, Australia, Africa, Germany, and South America. Smith and Kilbourne first named the blood parasite found in Texas fever pirosoma; later the organism was called apiosoma. Since, however, these family names had previously been applied to other organisms, the name was subsequently changed to Piroplasma bigeminum, which means pear-shaped protoplasmic twin bodies.

Pathologic Anatomy.—If the disease has taken a very rapid course the carcass may be full and rounded; if the animal has been sick for a number of days there is generally emaciation and evidence of rapid loss of weight. In fat cattle which have contracted the disease and died from it during or shortly after transit a deep orange hue of the subcutaneous and other connective tissues is one of the most characteristic postmortem findings; frequently the muscles show a deep mahogany yellow tint. In thin milch cows and Southern stock cattle the icteric discolorization of the tissues is often absent. The degree of icteric discolorization of the tissues depends upon the number of red blood corpuscles which have been destroyed by the infecting parasites and the amount of hemoglobin which has first gone into solution in the blood plasma and has subsequently been deposited in the tissues or excreted by the urine. Ticks are often found adhering to the skin of an animal dead from Texas fever, and here and in places where they have fallen off edematous and hemorrhagic patches are seen.

Microscopic examination of the blood shows a more or less marked diminution of the number of red blood corpuscles (oligocythemia). The decrease in red blood corpuscles from a normal of about 7,000,000 may be down to 2,000,000 or even much less. If properly studied and examined with a high power, numerous red blood corpuscles show the specific cause of the disease, the piroplasma.

The heart often shows subpericardial and subendocardial petechiæ and ecchymoses; occasionally the myocardium shows cloudy swelling and fatty degeneration. The lungs are at times somewhat congested, and may likewise show small hemorrhagic spots. The peritoneal cavity may show a slight amount of yellowish serum. The spleen is very much enlarged, of a dark brown color, and the pulp very soft. The liver is enlarged, and shows a mottled appearance on the cut surface; the centres of the lobules are yellowish, the periphery reddish. Frequently the whole liver shows an icteric color. The bile ducts are much congested with thickened bile and stand out as markedly as if they had been artificially injected. The gall-bladder is distended and filled with thickened bile, the appearance of which has been likened to masticated grass. The kidneys show hemorrhagic and edematous congestion and parenchymatous degeneration with widen-

ing of the cortical portion and cloudy swelling of the epithelia. Small hemorrhagic spots are often seen in the cortical and medullary portions and also in the mucosa of the renal pelvis. Petechiæ and ecchymoses are also found in the gastric mucosa, while the small intestines show congestion generally without hemorrhages, but the cecum and colon frequently show hemorrhages and are often of a deep red or purplish-brown color. The urinary bladder often contains much hemoglobin-stained urine, which may contain so much of the blood-coloring matter that it is of a port-wine color.

Microscopic examination of sections of the various organs shows the presence of numerous piroplasmata in the capillaries.

Diagnosis.—The diagnosis of the disease in typical acute cases is generally comparatively easy on account of the characteristic symptoms, including the bloody urine (hemoglobinuria), and it can be established beyond any doubt by a microscopic examination of stained blood specimens. These are best prepared in the following manner:

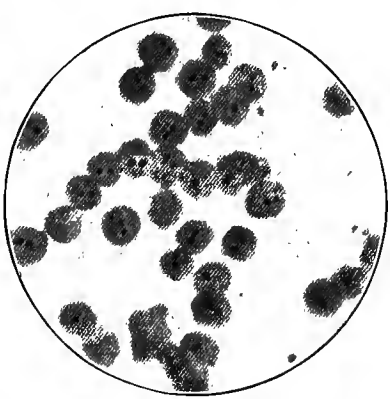
1. Clean an ear of the sick animal well with water and then with alcohol and dry it after the cleansing.

2. Make a small incision, with a scalpel, small pair of scissors, or with a large, sharp, triangular pointed needle.

3. Allow a drop of blood to flow on a glass slide, previously well cleaned with alcohol (so that there is no greasy matter on it).

4. With the margin of one end of another clean slide spread out the drop of blood in a thin, even layer on the first slide.

FIG. 203



Piroplasmosis of cattle (Texas fever). Smear from the kidneys of an animal dead from the disease. Hematoxylin-eosin stain. × 1000. (Author's preparation.)

5. Allow the slide to become air dry as quickly as possible. Drying may be hastened by waving the moist slide rapidly in the air.

6. Fix the blood film on the slide. The best method is to immerse it in absolute alcohol for twenty-five minutes or more.

7. Stain with Loeffler's blue. If instead of Loeffler's methylene blue a Romanowski stain or one of its modifications (Wright's stain) is to be used, it is best either not to fix the dry blood film at all or to fix it in pure methyl alcohol, which is the solvent of the stains of the Romanowski type.

Morphology of the Organism.—The Piroplasma bigeminum, or Babesia[1] bigemina, can, according to Smith and Kilbourne, be seen

[1] In honor of Babes, who first saw them.

in the fresh blood of cattle suffering from Texas fever as a pair of small, pale bodies, each one pear-shaped and touching the other, or directed toward each other with their narrow-pointed ends. They vary in size in different blood corpuscles, but the two forming a pair are generally fairly equal. They are about 2 to 4 micra long and 1.5 to 2 micra wide at the broader ends. The pointed ends touch or nearly touch each other and the axes of the two bodies form a varying angle; they may be almost parallel or they may form a straight line, and they may show any intermediate stage between these two extremes. The piroplasmata are of a homogeneous not granular appearance, and they are well differentiated from the stroma of the red blood corpuscles in which they are found. The smaller forms are generally perfectly homogeneous, the larger pear-shaped bodies often show at the periphery of the large end a small, highly refractive, somewhat darker round body of 0.1 to 0.2 micron in diameter, and also at or near the centre of the large end a round or oval body of 0.5 to 1 micron in diameter. Sometimes the piroplasmata in fresh blood show ameboid motion with a change in the contour of their body, but without the formation of any well-marked pseudopodia. Dead or dying organisms lose the pear shape and assume a round outline. If stained cover-glass or slide preparations are made from blood containing piroplasmata the alkaline anilin stain is seen to have been taken generally more intensely at the margin than in the interior. Not all parasites show the pear shape or occur in pairs; many are single, round, oval, or irregular.

According to Smith and Kilbourne, usually only 1 per cent. of the erythrocytes are infected, but shortly before death the number of infected corpuscles may be from 5 to 10 per cent. As the fever disappears the parasites likewise disappear from the blood. If the animal dies the blood in the capillaries of the internal organs often shows an enormous infection. The piroplasmata are most numerous in the kidneys (in 50 to 80 per cent. of the red blood corpuscles), and after them in the liver and spleen, and they are also found free in these organs between the red corpuscles. A few hours after the death of their host the parasites evidently in consequence of degenerative changes lose the pear shape and assume a round form. These degenerative or involution forms, however, are probably not dead parasites, since such blood retains its infective character, and since blood infected with piroplasma and obtained under aseptic conditions may remain virulent after a stay of sixty days in the refrigerator. In the incubator at 37° C. such blood remains virulent for only about one week. Smith and Kilbourne have also described coccus-like bodies, measuring from 0.2 to 0.5 micron, in 5 to 50 per cent. of the red blood corpuscles in the mild autumnal form of the disease in Texas cattle. These bodies can generally not be seen unstained, but only after treatment with methylene-blue solution. They are looked upon by the investigators named as a form in the

life cycle of the piroplasma, not as degenerative basophilic granules of the protoplasm of the red blood corpuscles.

Kossel and Weber studied the hemoglobinuria of cattle in Finland and confirmed the previous observations of Smith and Kilbourne, and were able, by the use of the Romanowski stain, to add further morphologic details. They found that the very smallest intracorpuscular parasites, which have about one-sixth of the diameter of the red blood corpuscles, are very delicate ring bodies, the margin of which stains red, the inner portions blue. Other small parasites are irregular in shape and show the beginning of an arrangement of the chromatic substance into two portions. In somewhat larger bodies the division of the chromatin into two parts has become quite distinct, while sometimes the chromatin is split up into four portions. These authors, however, were not able to demonstrate in piroplasma, asexually or sexually, dividing bodies as they occur in human and avian malarial parasites which form spores by two distinct types.

While nothing definite is known as to the propagation of the piroplasma in the body of infected cattle it is quite evident that it must take place in some way, since an enormous increase in the number of parasites within a short time can be observed in certain stages of the disease. Doflein has observed that the nucleus-like body in the interior of the parasites under some conditions breaks up into three, four, or more smaller fragments. He looks upon this process as an asexual spore-formation (schizogony), and he considers the large pear-shaped bodies as sexual forms (gametocytes).

Whether the piroplasmata found in animals of the cattle tribe in various parts of the world are one identical species, or whether they are varieties or distinctly different species, is a question which cannot as yet be decided definitely. In East Africa, Robert Koch found bacilli-like forms, often four in one corpuscle, in a very large percentage of the red blood corpuscles in the fatal hemoglobinuria of cattle. These rods by curving upon themselves formed ring-like bodies; they were generally thicker in the middle than at either end, and by intermediate forms gradually lead to the typical shape of the piroplasma. Since such forms have never been seen in Texas fever in the United States, this East African piroplasmosis is perhaps due to a species different from Piroplasma bigeminum.

Animals Susceptible.—The piroplasmosis of cattle in America is not transmissible to other animals. Experiments have been made upon horses, asses, sheep, rabbits, guinea-pigs, dogs, cats, pigs, mice, rats, and chickens. The tests were all negative. If, however, blood from an animal sick with Texas fever is inoculated by any one of a variety of methods, such as intravenous, subcutaneous, intraperitoneal, intramuscular, intracerebral, into a healthy head of cattle a marked elevation of the temperature takes place, after three to seven days, and piroplasmata may be seen in the circulating blood. After a few more days the number of red blood corpuscles and the hemoglobin

become diminished and the urine in grave cases assumes a dark color. The best method for making the inoculation experiments is to use 5 to 10 c.c. of defibrinated blood from an infected animal.[1]

Animals infected with such blood may acquire a fatal infection, or the infection may take a moderately severe or even a mild course.

Natural Mode of Transmission.—Texas fever, in the natural course of events, always is transmitted from an infected animal to a healthy one by blood-sucking ticks. The tick which acts as the intermediate host of the Piroplasma bigeminum in this country is called Boöphilus bovis, Rhipicephalus annulatus, or Margaropus annulatus. In northern Europe piroplasmosis of cattle is spread by the tick, known as Ixodes reductus. It is claimed that this tick also occurs in America, and may here be concerned in the spreading of Texas fever. The biting and sucking ticks probably discharge with their saliva an irritating fluid which produces the local hyperemia and which also introduces the protozoan parasites into the bitten animal. This discharge of piroplasma from the intermediary host (the tick) to cattle is similar to that of the Plasmodium malariæ by mosquitoes (anopheles) into man. The developmental stages of the Plasmodium malariæ in the mosquito, however, are well known, while nothing is known of such stages of piroplasma in cattle ticks. Mosquitoes do not transmit the malarial parasites through their ova to their offspring, but the eggs of a tick that has fed upon infected cattle will develop ticks that spread the disease.

A knowledge of the cattle tick, its life history and habits, is necessary in the campaign to limit and exterminate the disease, and for this reason the description given by Graybill in *Farmer's Bulletin No. 378*, United States Department of Agriculture, Washington Government Printing Office, 1909, is here inserted:

"In tracing the life history of the cattle tick it will be convenient to begin with a large, plump, olive-green female tick, somewhat more than half an inch in length, attached to the skin of the host. During the few preceding days she has increased enormously in size as a consequence of drawing a large supply of blood.

"When fully engorged she drops to the ground, and at once, especially if the weather is warm, begins to search for a hiding place on moist earth beneath leaves or any other litter which may serve as a protection from the sun and numerous enemies. The female tick may be devoured by birds or destroyed by ants, or may perish as the result of unfavorable conditions, such as low temperature, absence or excess of moisture, and many other conditions; so that many which fall to the ground are destroyed before they lay eggs.

"Egg-laying begins during the spring, summer, and fall months,

[1] Blood is defibrinated in the following manner: Allow blood drawn under aseptic precautions to run into a sterile vessel containing some glass beads or fragments of glass. Shake well for some time. The fibrin collects around and clings to the glass pearls, etc., and the defibrinated blood may then be poured off into another sterile vessel.

Fig. 206

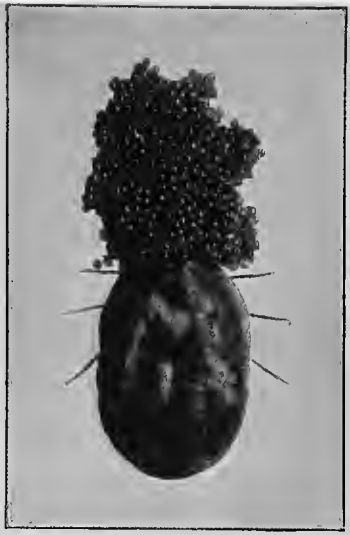


Fig. 207

Fig. 208

Fig. 209

Fig. 210

Figs. 204 to 210.—Cattle ticks in various stages. Fig. 204. Full-grown female tick, engorged and ready to drop to ground and deposit eggs. (Magnified 3 times.) Fig. 205. Tick laying eggs. One tick may lay as many as 5000 eggs. (Magnified 3 times.) Fig. 206. Larvæ or seed ticks after emerging from eggs. (Magnified 9 times.) Fig. 207. Young ticks before (*a*) and after (*b*) first molt. At this stage the ticks have attached themselves to a host (cow, steer, etc.), and have changed from a brown color to white. It will be noticed that the tick has six legs before molting and eight afterward. (Magnified 9 times.) Fig. 208. Young tick nearly ready to undergo the second molt. The tick at this stage is known as a nymph. (Magnified 6 times.) Fig. 209. Male tick. (Magnified 6 times.) Fig. 210. Female tick after second molt. This tick is now sexually mature and slightly larger than the male, but will later greatly increase in size until ready to drop to the ground and deposit eggs. (Magnified 6 times.) (Graybill.)

in from two to twenty days, and during the winter months in thirteen to ninety-eight days. The eggs are small, elliptical-shaped bodies, at first of a light amber color, later changing to a dark brown, and are about one-fiftieth of an inch in length. As the eggs are laid they are coated with a sticky secretion which causes them to adhere in clusters, and no doubt serves the purpose of keeping them from drying out. During egg-laying the mother tick gradually shrinks in size and finally is reduced to about one-third or one-fourth of her original size. Egg-laying is greatly influenced by temperature, being retarded or even arrested by low temperatures. It is complete in from four days in the summer to one hundred and fifty-one days during the fall and the beginning of winter. During this time the tick may deposit from a few hundred to more than 5000 eggs. After egg-laying is completed the mother tick has fulfilled her purpose and dies in the course of a few days.

"After a time, ranging from nineteen days in the summer to one hundred and eighty-eight days during the fall and winter, the eggs begin to hatch. From each egg issues a small, oval, six-legged larva, or seed tick, at first amber colored, later changing to a rich brown. The seed tick, after crawling slowly over and about the shell from which it has emerged, usually remains more or less quiescent for several days, after which it shows great activity, especially if the weather is warm, and ascends the nearest vegetation, such as grass, herbs, and even shrubs.

"Since each female lays an enormous mass of eggs at one spot, thousands of larvæ will appear in the course of time at the same place and will ascend the near-by vegetation and collect on the leaves. This instinct of the seed tick to climb upward is a very important adaptation to increase their chances of reaching a host. If the vegetation upon which they rest is disturbed they become very active and extend their long front legs upward in a divergent position, waving them violently in an attempt to seize hold of a host.

"The seed tick, during its life in the pasture, takes no food, and consequently does not increase in size, and unless it reaches a host to take up the parasitic portion of its development it dies of starvation. The endurance of seed ticks is very great, however, as they have been found to live nearly eight months during the colder part of the year.

"The parasitic phase of development begins when the larvæ or seed ticks reach a favorable host, such as a cow. They crawl up over the hair of the host and commonly attach themselves to the skin of the escutcheon, the inside of the thighs and flanks, and to the dewlap. They at once begin to draw blood and soon increase in size. In a few days the young tick changes from a brown color to white, and after from five to twelve days sheds its skin. The new form has eight legs instead of six, and is known as a nymph.

"In from five to eleven days after the first molt the tick again

sheds its skin and becomes sexually mature. It is at this age that males and females are with certainty distinguishable for the first time. The males emerge from the skin as brown, oval ticks, about one-tenth of an inch in length. He has reached the limit of growth and goes through no further development. Later he shows great activity in moving about over the skin of the host. The female at the time of molting is slightly larger than the male. She seldom shows much activity, seldom moving far from her original point of attachment. She still has to undergo most of her growth. After mating the female increases very rapidly in size, and in from twenty-one to twenty-six days after attaching to a host as a seed tick she becomes fully engorged and drops to the ground of the pasture, to repeat the cycle of development.

"To sum up, on the pasture there are found three stages of the tick—the engorged female, the egg, and the larva; and on the host (cattle) are found four stages—the larva, the nymph, the sexually mature adult of both sexes, and the engorged condition of the female.

"In undertaking measures for eradicating the tick it is evident that the pest may be attacked in two locations, namely, on the pasture and on the cattle.

"In freeing pastures the method followed may be either a direct or an indirect one. The former consists in excluding all cattle, horses, and mules from pastures until all the ticks have died of starvation. The latter consists in permitting the cattle and other animals to continue on the infested pasture and treating them at regular intervals with oils or other agents destructive to ticks and thus preventing engorged females from dropping and reinfesting the pasture. The larvæ on the pasture, or those which hatch from eggs laid by females already there, will all eventually meet death. Such of these as get upon the cattle from time to time will be destroyed by the treatment, while those which fail to find a host will die in the pasture from starvation.

"Animals may be freed of ticks in two ways. They may be treated by solutions, etc,. that will destroy all the ticks present, or they may be rotated at proper intervals on tick-free fields until all the ticks have dropped."

Epidemiology.—A number of points in the epidemiology of Texas fever, formerly quite mysterious and unexplainable, are now easily understood, since the etiology of the disease has been cleared up. Wherever Texas fever or piroplasmosis of cattle has occurred it was observed that animals on the pastures are more commonly attacked than animals kept in barns. It was also noticed long ago that a wet, marshy ground upon which cattle entered in spring formed a favorable soil for the appearance of the disease. Hot weather favors outbreaks more than a cool temperature. Animals born and raised in infected territories are much more resistant than animals born and raised in a free territory and later brought to the

infected territory. This is due to the fact that very young animals are not very susceptible to the disease; they acquire it in a mild form and a certain degree of immunity becomes established by repeated annual infections. The immunity so acquired, however, is no real immunity, but simply consists in the presence of a small number of piroplasmata and a tolerance against them. If such animals are exposed to fatigue (in transit) or to other diseases they may develop a malignant outbreak of the disease.

Such partially immune animals if brought to a non-infected district may become the source of violent outbreaks among the animals of the hitherto free territory. Transmission in these cases may be brought about by either one of two ways: (1) The animals from the infected district may carry with them infected ticks which will directly spread the disease in a hitherto free territory, or (2) they may not bring ticks along, but find in their new surroundings ticks which can and will spread the disease.

Immunization of Cattle.—It was noticed by Smith and Kilbourne that animals after recovery from an attack of Texas fever in one year were comparatively immune against new attacks in subsequent years in spite of being much exposed to infected ticks. Schroeder was one of the first in this country experimentally to inoculate young northern cattle with blood from infected Southern animals, producing by this method a mild attack of Texas fever. Subsequently he exposed the inoculated animals together with non-protected control animals in the South to the natural tick infection. The results were very favorable and promising: most of the protected animals lived, and all the controls died.

Dalrymple[1] has published a report on the results and experiences of protective inoculation of cattle against Texas fever. He states (1) that sterile blood serum of infected animals obtained by centrifuging the blood and separating the corpuscles from the serum, has no value whatever in immunizing animals; (2) that susceptible cattle may be immunized by infecting them with piroplasma through the medium of infected seed ticks, but on account of certain troublesome conditions the method is not as practical as could be desired. The results of experiments to utilize the blood from ticks in immunizing inoculations is summarized as follows by Dalrymple:

"The blood with which the adult ticks are filled, after maturing on Southern cattle, carries with it the power to produce Texas fever when injected under the skin of a susceptible animal.

"Experiments indicate that we may be able to take ticks from recently immunized animals, ship them considerable distances, and utilize them as a substitute for the blood drawn from the vein, where recently immunized animals are not available.

"Experiments further indicate that this will give a milder form

[1] Louisiana State University and A. and N. College Bulletin, No. 84, October, 1905.

of the disease, and afterward, immunty just as effectual as when the blood is taken from an immune animal immediately before being used.

"We have not as yet found any way of preserving the blood drawn from the vein for any considerable time without its losing its power to produce immunity."

The Louisiana report of Dalrymple gives the following conclusions and directions as to the immunizing of Northern cattle by the use of fresh blood from infected animals:

"Previous to the discovery and adoption of the blood-inoculation method of immunizing susceptible Northern cattle against the ravages of Texas fever the mortality in these animals ranged anywhere from 40 to 90 per cent. This, necessarily, discouraged Southern stockmen in the importation of pure-bred cattle for the purpose of improving their herds, and accounts, mainly, for the scarcity of pure and highbred stock in the South up to within recent years.

"Consequent upon the use of this artificial method of immunization, however, the death rate from the fever has been enormously reduced. In a bulletin issued by the Texas Experiment Station in 1902 a record was compiled showing the percentage mortality of inoculated cattle that had been treated at the Texas, Louisiana, and other Southern (including Missouri) stations, which comprised several thousand head (4562 up to January 1, 1904), to be only 7.7, and that, too, under various conditions of treatment after they had been placed in their owner's care. This record has given increased encouragement to cattle men in the South.

"The technique of the operation as practised in the Louisiana Experiment Station is the following: The supply animal from which the immunizing infected blood is used is either a native or a Northern immune which should be in robust health and condition. Experiments and experience seem to indicate that the most suitable subjects for immunization are cattle from eight to twelve months old, in good flesh, and weight from 500 to 800 pounds. Before inoculation it is well to allow the animal to rest for a few days, especially those that may have come off a tedious railroad journey; and during this time they should be well and carefully fed and kept absolutely free from ticks.

"The operation seems more easily performed with the supply animal thrown down and tied. The hair is clipped from a portion of the skin of the neck just over the jugular vein. The denuded part is bathed with an antiseptic solution. The neck of the animal is now straightened and tensed, and a piece of strong cord or small rope tied around its base sufficiently tight to check the flow of blood and raise the vein. A small trocar and cannula, or hollow sharp-pointed needle, which has previously been sterilized, or disinfected, is then inserted into the distended vein and is directed up the vessel toward the head. As soon as the needle enters the vein the blood

passes through it into a sterilized glass, and when sufficient blood has been obtained the needle or cannula is withdrawn, the rope around the neck loosened, and the wound bathed with antiseptic solution.

"To prevent the blood from clotting, after it has been withdrawn it is immediately stirred slowly with a thin glass rod, which has been disinfected, until as much as possible of the clot has collected on it, and it is then withdrawn. The remaining defibrinating blood, or a part of it, is then drawn up into a clean hypodermic syringe, and the quantity to be injected gauged by a small screw-regulator on the piston.

"The animal to be inoculated is prepared by clipping the hair from off a portion of the skin—about the size of the hand—behind or a little above the point of the elbow, on the side of the chest; any part where the skin is loose and thin will answer. This is disinfected as in the case of the supply animal. The skin is then drawn out between the thumb and forefinger of the left hand and an incision made through it with a narrow, sharp-pointed knife or lancet, to allow of easy introduction of the hypodermic needle, care being taken not to injure the chest wall. If the needle is a strong one it may not be necessary to use the lance. The syringe is now attached to the needle and the required amount of blood injected under the skin. After withdrawal of the needle the part is again lightly bathed with the antiseptic solution. Success depends very largely upon the antiseptic precautions taken in the operation. Consequently, all instruments and utensils, the hands of the operator and the operative area of skin should be thoroughly disinfected.

"The standard amount of blood used at the Louisiana Station for some time has been 1 cubic centimenter (about 16 drops) for animals of any age. Latterly, however, it has been customary to administer a second dose of 2 cubic centimeters after recovery from the fever period. The object is to increase if possible the degree of immunity before the animal is exposed to ticks. After the second injection of blood the patient is kept under observation for ten days or somewhat longer, and the temperature taken to watch the course of the fever, should there be any.

"We have previously stated that the blood before injection was stirred to remove the clot (defibrinated). This is usually done when a number of animals are to be inoculated, to prevent clotting before the work is completed. In the case of a single animal, however, or even two or three, the blood may be drawn directly from the vein of the supply animal into the hypodermic syringe and injected immediately into the other animal or animals."

Although inoculation may be performed at any season of the year, the best time in the Southern climate is during the late fall or early winter months. This prevents a too sudden gross infection with ticks when the animal is turned into pasture, as would naturally be the case during the summer months.

Kaumanns, in a paper read before the 1909 (Chicago) Meeting of the American Veterinary Association, and published in full in the Proceedings of the Association, strongly advocates the crossing of American cattle with Brahma cattle from India for the purpose of producing a natural immunity. He states that only 35 per cent. of Indian blood is required to produce a race almost entirely immune against Piroplasma bigeminum infection. The great difficulty in carrying out such a plan consists in getting Indian cattle free from latent trypanosoma infection. In attempts made in the past it was found that a high perentage of such cattle, though examined thoroughly before exportation from India, were found infected with surra upon arrival in the United States. Evidently the journey and the change of climate awakened the latent trypanosomiasis.

PIROPLASMA CANIS.

The piroplasma infection in dogs was first described by Piana and Galli-Valerio in Italy, in 1895. The two investigators recognized the similarity between the parasites in the dog's blood corpuscles and those in Texas fever, and called them Piroplasma bigeminum, var. canis. They were subsequently found in dogs by a number of authors and their morphology has been studied particularly by Schilling, Nuttal and Graham, Kinoshita, Bowhill, LeDuc, Christopher, and others. In several respects they are the best-known representatives of the family piroplasma or babesia.

In the fresh blood of the dog the intracorpuscular parasite can best be seen at the height of the fever; it appears in the inside of the erythrocyte as a light, highly refractive, sharply defined body, generally found in somewhat enlarged red blood corpuscles. If examined in fresh blood on a warm stage the protoplasm of the parasite shows some contractility and appears to send out fine processes. The latter may be so fine that they look like flagella. The largest forms of the parasites are found in freshly infected dogs, the smallest in old chronic cases. Blood smears stained with Romanowski's, Wright's, or Giemsa's stain show a small amount of oval or pear-shaped, round or ring-shaped protoplasm stained bluish with a dot stained red. The latter is the nucleus of the parasite. The nucleus is not always small, sometimes it is quite large, and occupies a considerable portion of the cytoplasm of the piroplasm. These forms, after the fever has reached its height, and when the temperature is going down, are also found outside of red blood corpuscles in the blood plasma. The microörganism is also found in the internal organs, particularly in the liver, the kidneys, and the bone marrow.

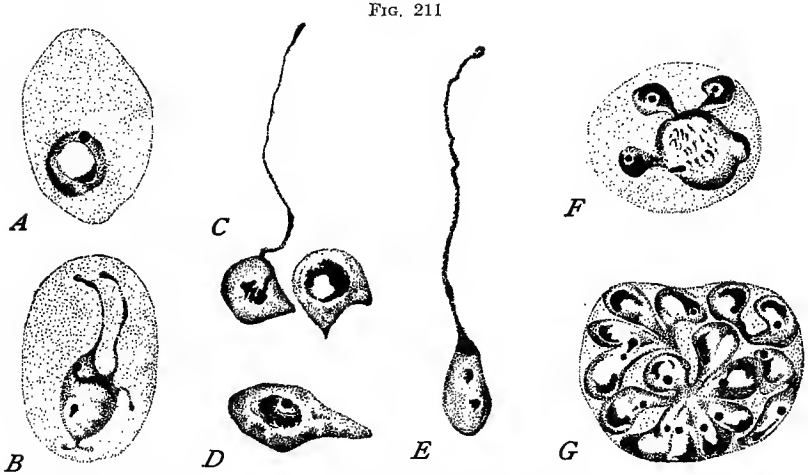
The nucleus of Piroplasma canis is of indefinite shape, composed of chromatin granules varying in form; it is generally situated eccen-

trically in the intracorpuscular forms, but in the centre of the parasites which are found free in the blood plasma.

The Piroplasma canis, like the bigeminum, reproduces in the interior of red blood corpuscles. According to Nuttall and Smith a small, round, young form is first present. This becomes larger, begins to divide by becoming somewhat saddle-shaped and pear-shaped, with the twin arrangement later on. The twins after being completely divided leave the red blood corpuscles, enter new ones, and repeat the cycle.

Kinoshita has described an irregular division or budding process which he regards as an asexual multiple reproduction (schizogony), with merozoite formation, as it occurs in malarial organisms. Christopher, however, claims that such a mode of division does not occur in piroplasmata, that they divide always into two equal halves, but that subdivision may start before the first division is complete.

FIG. 211



Stages in the development of Babesia canis. (After Kinoshita.) *A*, round discoid parasite in a blood corpuscle; *B*, ameboid form, with long processes; *C*, a pair of "mature" gametes; *D*, a mature "female" gamete; *E*, a mature "male" gamete; *F*, a budding form in blood corpuscles; *G*, a group of sixteen "young" gametes.

The most constant symptom in canine piroplasmosis is hematuria, just as in Texas fever of cattle.

Natural infection is brought about by biting ticks. Several kinds have been named as being the intermediate host of Piroplasma canis, among them Hemophalis leachii, which, however, can only transmit the disease during the mature stage; neither the six-legged larvæ nor the eight-legged nymphæ can transfer the disease from infected to healthy dogs.

PIROPLASMA OVIS.

Sheep are subject to a disease known as icterohematuria, or hemoglobinuria. It is, like the preceding affections of cattle and dogs, a piroplasmosis. The intracorpuscular organisms were first seen by Babes in 1888, who, however, did not recognize their protozoan nature. Piroplasmosis in sheep was subsequently described by Bonome in Italy, Nicolle in Turkey, Leblanc in France, and Hutcheson in Transvaal. The most marked pathologic changes in piroplasmosis in sheep are a serous infiltration of the subcutaneous, retroperitoneal, and mediastinal connective tissue, inflammatory and hemorrhagic changes in the gastro-intestinal tract, enlargement and great congestion of the spleen, parenchymatous degeneration and necrosis of the liver, parenchymatous degeneration of the kidneys, and multiple subserous and submucous hemorrhages. The bladder contains a bloody urine. The parasites causing the disease are piroplasmata of the usual shape and structure. Dividing forms can best be seen in juice expressed from the spleen. The anemia caused during the short course of the disease is very profound, and the count of erythrocytes sinks from 8,000,000 to 1,500,000. The mortality of the disease is very high; recovered animals are said to be immune. The disease is spread by ticks.

PIROPLASMOSIS OF HORSES.

Besides the infectious anemia in horses, which is a disease evidently due to an ultramicroscopic filterable organism concerning which absolutely nothing is known, anemias in horses due to piroplasma infection have been observed at various times and places. Ziemann claims to have seen intracorpuscular piroplasmata in the blood of horses sick with hematuria in Germany. Hutcheson reported similar findings from Cape Colony. Bordet and Danysz found equine piroplasmosis in Transvaal. Some authors have claimed that the Plasmodium malariæ has also been found in horses, but this is denied by Laveran, who holds that all intracorpuscular parasites found in the blood of horses are piroplasma and none Plasmodium malariæ. Some of the later observations on equine piroplasmosis were made by Pallin in India and Robert Koch and Theiler in Africa. The latter distinguishes two types: a mild form, where a diagnosis can only be made by finding a few piroplasmata in the blood, and serious cases, with high fever and marked sickness. The former type generally ends in recovery, the latter in death. The parasites are found in the blood and most abundantly in the spleen. The organisms are generally round, have a diameter from 0.5 to 2.5, and look a good deal like tropical and tertiary malarial parasites. Pear-shaped forms

in couples are, however, also seen. Romanowski's stain shows a red-stained chromatin granule near the periphery of the parasites; pigment (as in malarial parasites) is never seen. Laveran described amitotic division of the nucleus; two divisions may follow each other rapidly, so that four parasites arranged in rosette form may be seen in a red blood corpuscle. The most prominent changes in equine piroplasmosis are intense jaundice, with enormous enlargement of the spleen, which may weigh ten pounds or more. The pulp of the spleen is very soft, dark, and tar-like. The bladder contains hemorrhagic urine. Subserous and submucous hemorrhages are frequently seen; the heart muscle is very soft and flabby, and ruptures easily. The disease is spread, like the other piroplasmoses, by ticks.

QUESTIONS

1. What kind of a disease is Texas fever?
2. What other names have been given to this disease?
3. What were the claims of Babes as to the nature and cause of the disease?
4. What is the real cause of Texas fever? Who discovered it?
5. Describe the pathologic changes in a very acute case of Texas fever.
6. What are the blood changes in Texas fever?
7. What is the meaning of the terms oligocythemia and oligochromemia?
8. How can an absolute diagnosis of Texas fever be made?
9. Describe the steps in a blood examination for establishing a diagnosis of Texas fever.
10. Describe the morphology of the Piroplasma bigeminum.
11. What is the relation between the parasites and febrile temperatures in infected animals?
12. Where are the piroplasmata found most numerous after the death of an infected animal?
13. How long does blood infected with piroplasmata remain virulent under various conditions?
14. Describe the cultural properties of Piroplasma bigeminum and the preparation of the proper culture medium.
15. What is the natural mode of transmission in Texas fever?
16. What animals are susceptible to this disease?
17. How can the disease be transferred artificially?
18. What are the technical names of the Texas fever cattle tick? Describe its life history.
19. What methods have been practised to immunize cattle against Texas fever?
20. Describe in detail the Louisiana method.
21. When is the best time to immunize animals against Texas fever?
22. What animal is the host of Piroplasma canis? Describe the morphology of the latter.
23. Describe its method of reproduction.
24. Discuss the different views as to processes of reproduction in Piroplasma canis.
25. What is the most constant symptom in piroplasmosis of dogs?
26. How is the disease spread?
27. What difference in the mode of spreading is there between piroplasmosis of dogs and Texas fever?
28. Describe the most prominent pathologic changes of ictero hematuria in sheep. What is the cause of the disease?
29. What are the most prominent pathologic changes in piroplasmosis of horses? Where has the disease been observed?

CHAPTER LV.

RABIES AND THE NEGRI BODIES (NEURORYCTES HYDROPHOBIÆ).

RABIES, lyssa, hydrophobia, canine madness, "Wasserscheu," "Tollwuth," "Hundswuth" (German), "rage" (French), is an acute contagious, generally fatal disease of wolves, foxes, dogs, and more rarely of other domestic animals and man. It is due to a specific virus, which, with the infective saliva, gains entrance into the body of a susceptible being through a wound generally caused by the bite of some animal suffering from the disease.

Historical and Occurrence.—Rabies among dogs and the danger to other animals from the bite of a rabid dog were known to Aristoteles, the Greek naturalist and philosopher. That the saliva of such animals was the carrier of the infective agent was shown experimentally by Zinke, Gruner, and Salm in the early part of the last century. Galtier, in 1879, was the first to inoculate rabbits, and in 1881 Pasteur and his co-workers, Roux, Chamberland, and Thuilliers, became the main exponents in the modern study of hydrophobia upon which is based our exact knowledge of the disease and the methods of its prevention by the inoculation of an attenuated virus. The disease has been encountered almost all over the world, but has apparently been kept out of Australia. It has been on the increase during the last decade or two in the United States.

Natural Infection.—Natural infection, as a rule, is caused by the bite of animals suffering from the disease, but sometimes dogs in the early stages of unrecognized rabies have inoculated persons by licking a place where there is a small abrasion of the skin. The saliva is most infective after the disease has well developed and during its subsequent course, but may also be infective before any symptoms of rabies appear. The danger of the bite from a rabid animal depends upon the greater or lesser virulency of the saliva, upon the extent of the wound, the amount of laceration, the vascularity and nerve supply of the tissue, and upon the greater or lesser distance of the wound from the central nervous system. The less the distance the greater the danger; hence, wounds of the face or head are particularly dangerous. Horses and cattle are especially liable to contract hydrophobia if bitten by rabid dogs, wolves, or foxes in the lips, cheeks, or nose. The danger of the bite is much lessened if the parts are covered by a dense fur, or, in the case of man, by heavy clothing. It has been shown that shorn sheep are much more liable to develop rabies after being bitten than those covered with a dense wool. The virus,

so far as known, cannot penetrate the intact skin, but rabbits may be infected through the intact nasal and conjunctival mucosa. The virus cannot enter through the gastro-intestinal tract, as has been shown by Nocard in his feeding experiments. The percentage of infections in animals bitten by dogs suffering from rabies is variously estimated, and the figures given extend over a wide range, anywhere from 5 to 40 per cent. There appears to be both a racial and an individual variability. The virus of hydrophobia after having gained access to the body of a susceptible animal travels from the portal of entrance to the central nervous system, where it spreads gradually. According to the investigations of Vestea and Zagari, inoculation into the sciatic nerve of the rabbit is first followed by paralysis of the hind leg of the same side and the paralysis progresses from behind to the anterior part of the body. When the inoculation is made in an anterior extremity the progress is from in front backward. The virus multiplying in the central nervous system affects the vessel walls and produces around them small, round-celled inflammatory infiltrations. It damages the ganglion cells, causing psychical disturbances, increase of reflex irritability, and later degenerative manifestations, with paralysis, which finally affects the respiratory apparatus and so leads to death.

Period of Incubation.—One of the most remarkable characteristics of hydrophobia is the great variability of the period of incubation after natural infection. As a rule, this period lasts a few weeks, but not infrequently may be prolonged to a few months. In dogs and hogs the period of incubation is frequently shortened to ten to fifteen days; in horses and cattle it is frequently from one to three months. According to statistics by Roell, of 144 rabid dogs, 43 per cent. developed manifest symptoms of hydrophobia within thirty days after being bitten, 40 per cent. between the thirtieth to the sixtieth day, 14 per cent. between the sixtieth to the nintieth day, and 3 per cent. between the fourth to twelfth month. Zuendel gives the following figures for 579 head of cattle: 5 per cent. in less than fifteen days, 23 per cent. between fifteen and thirty days, 39 per cent. between thirty to forty-five days, 13 per cent. between forty-five to sixty days, 17 per cent. between three to six months. One animal after forty-two and one after ninety-five weeks. Unusually long periods of incubation in horses have been reported; by Gosswinter, twenty months; by Bahr, twenty-one months; by Swain, twenty-five months; in cattle by Szabo, three hundred and twenty-three days; Mieckley, three hundred and twenty-seven days; Leipert, nearly twenty months; Kalt, twenty-three months. Lignière reported the case of a rabid watch dog on a farm biting twenty head of cattle. Four oxen out of the twenty animals bitten died after incubation periods between two and six months. All four succumbed to the paralytic form of the disease, as also did one cow, but only after an incubation period lasting three years In man the period of incubation likewise varies considerably. Paltauf,

who has recently furnished a very interesting contribution to the pathogenesis of rabies in man, states that the shortest period on record is about fourteen days; the average period, eight to twelve weeks. This author has seen a case with a period of incubation of twenty months; Spencer one of two years, four months, and Krikoff one of three years and two and one-half months. While all fully developed cases of rabies in man lead to a fatal issue, man is not very susceptible to the disease. According to older statistics dealing with persons bitten by rabid dogs, 16 to 20 per cent. develop the disease. Hoegyes gives a percentage figure of 13.9, other authors of 5 to 6 per cent. Kirchner's figure for Germany is 2 per cent. to 3 per cent., and Paltauf thinks that the figure is certainly below 10 per cent. These percentages all refer to persons bitten by rabid dogs. The more serious lacerations produced by rabid wolves are much more dangerous, and it is estimated that 60 per cent. of persons bitten by these animals contract the disease and die from it if not treated.

Paltauf's recent studies of the pathology and pathogenesis of rabies in man were made on four persons who died shortly after being bitten by rabid dogs from some intercurrent diseases which had nothing at all to do with hydrophobia.

In all four cases it was found that the medulla, when emulsified and injected subdurally, infected rabbits with rabies, showing the presence of active virus in the patients' nervous tissues; but this virus was in an attenuated condition, since the incubation period in the inoculated rabbits was unusually long, forty days and over, and the type of rabies developed was that of the chronic or "consumptive" form. As probably at the highest estimate but one person in ten of those bitten develops rabies, it must be assumed that four consecutive positive findings do not represent latent infection which would have manifested itself had the patients lived longer. It appears rather that these observations indicate that in persons bitten by rabid animals the virus commonly reaches the central nervous system, but that nine times in ten it is there destroyed by the natural defensive agencies without causing symptoms. These agencies may be made more effective by the immunizing process of the Pasteur treatment. In other words, rabies-inoculated men usually develop a latent infection which is overcome without the symptoms of rabies. The medulla of three other persons, who died shortly after the completion of a course of Pasteur treatment, were found to be non-infectious for rabbits, indicating that the virus was destroyed under the influence of the immunization.

Presumably the virulency of the virus with which the individual is inoculated is one of the main factors in deciding whether it will be overcome or not, for the bites of rabid wolves cause rabies in about 60 per cent. of those bitten as against from 6 to 10 per cent. or less of fatalities from dog bites, and none from subcutaneous inoculation of attenuated rabbit virus. Possibly in other cases the failure

to destroy the virus may depend on individual lack of immunizing power, and sometimes on local disturbances in the central nervous system. Medical literature is full of records of cases in which either physical or mental shock seemed to determine the development of rabies.

Paltauf, from his studies and the observations given above, draws the following summary of conclusions with reference to the behavior of the virus of rabies after its entrance in natural infection into the bodies of animals and persons:

1. The period of incubation in rabies in man varies between two weeks and two years and over.

2. Dogs are evidently quite susceptible to the virus of rabies, though not highly so, since about 40 per cent. of dogs bitten contract the disease.

3. Man is not very susceptible to the virus of rabies in dogs; only from 6 to 9 per cent. or perhaps even a much smaller number of persons bitten develop rabies, and if not properly treated early, die from the disease.

4. Of persons bitten by wolves about 60 per cent. develop rabies.

5. There is an enormous difference between the original susceptibility of man to the virus of rabies in dogs and the mortality in cases which have proved to be susceptible.

6. Observations made on four persons bitten by rabid dogs and who died from intercurrent diseases (not rabies) soon after the Pasteur treatment was begun showed that their cords when emulsified and injected into rabbits produced after long periods of incubation the slow, so-called "consumptive" form of rabies.

Paltauf does not try to explain the long period of incubation of two to three years and more which has been observed in a few authenticated cases of rabies in animals and persons. It might be explained, perhaps, as follows: When animals or persons are bitten by rabid dogs there may be a shorter or longer period of latency, and the virus may finally be completely exterminated by the protective powers of the body. On the other hand, there may occur an incomplete destruction of the virus and imperfect immunity, but a continuous period of latency as is found in trypanosomiasis and particularly in Texas fever. In the latter disease a few piroplasmata may persist in cattle without detriment to the animal until a great strain, changes in environment, or intercurrent disease leads to a sudden multiplication of these organisms, with a typical explosive outbreak of the disease. Likewise, it is quite possible that the rabies organisms may survive for a long time in small numbers in a person bitten by a mad dog, and that under certain conditions they may suddenly multiply and produce a typical fatal attack of the disease.

Symptoms in the Dog.—The symptoms of rabies in the dog are described by Hart as follows:

"The symptoms are generally given for two different types, the

furious or irritable, and the dumb or paralytic. The latter type is always seen in the terminal stages of the former; and when the cases are of the dumb form from the outset it is probable that the toxemia is overwhelming, and such cases usually run a more rapid fatal course.

"*The Furious Type.*—In the furious type, following the variable period of incubation, there is first noticed a change in the disposition of the animal which should at once excite suspicion. Playful animals become morose and quiet, and reserved dogs may become unusually affectionate. The animal is nervous and easily excited, but obeys any command of its owner. In the course of a day or two the nervous condition increases and the animal becomes irritable and may snap if approached suddenly or startled. The bark becomes changed to a long-drawn-out combination between a whine and a howl, impossible to describe, but never forgotten when once heard. Some dog owners speak of it as being somewhat of the nature of the bark of a foxhound while in the hunt, but this does not properly describe it. The animal if loose may pick up and swallow straw, sticks, stones, leather, and other foreign bodies."

In some cases there is a tendency to bite parts of the skin, usually at the point where the animal was bitten, and in one case observed by Hart the animal chewed the skin over the os calcis until the entire head of the bone was exposed to view.

"There is a marked tendency in these early stages for the animal to seek quiet spots and to hide in corners or dark places. If an attempt is made to remove the animal, the person is in great danger of being bitten. The restlessness of the animal becomes more marked. He may stand looking intently into space as if at an imaginary object. There is difficulty in swallowing, and saliva may dribble from the mouth. The irritability increases until the animal becomes furious, biting at a stick or other object thrust toward him. At this stage, if the animal is not secured, he may leave home and travel for miles. During the long journey he will fight with dogs and attack other animals in his path, but never barks or makes any outcry during these attacks. The animal may go twenty to twenty-five miles from home, but always returns, if not prevented, in an exhausted condition, covered with wounds and dirt and greatly emaciated. Signs of commencing paralysis now appear, with drooping of the lower jaw, inability to swallow, and irregularity in the pupils. The legs become paralyzed and the animal passes into the dumb form of the disease.

"*Dumb Rabies.*—This form of the disease occurs in only a small percentage of the cases. The symptoms are somewhat similar to those of furious rabies, except that marked irritability is absent and there is an early appearance of paralysis. This form of the disease, therefore, renders the dog less dangerous than the furious type. The animal lies quietly in some secluded place and appears to be stupid. The paralysis of the jaw comes on early, the tongue protrudes and becomes congested and covered with dirt, giving rise to

the term 'black tongue,' which is used in some localities, especially in the South, and a bad synonym for this form of the disease. The use of the term should be discouraged, as it tends to confound the disease with dog distemper. The hind legs, trunk, and forelegs become paralyzed, and death usually ensues in about three days while the furious type lasts from six to eight days.

"Recovery from rabies in the dog after well-marked symptoms have developed is possible, and authentic cases have been reported by Pasteur, Roux, Babes, Courmont, Lignière, and Remlinger. This is so rare, however, that it is of little importance except in cases where a person has been bitten by a dog showing all the symptoms of rabies and the animal afterward recovered. The saliva in such cases remains virulent for several days or a week after the subsidence of symptoms, and a diagnosis can be made by inoculating rabbits with some of the salivary secretion."

The *consumptive form* of the disease mentioned above develops sometimes after the inoculation, natural or artificial, of a virus of a low degree of virulency. This form, perhaps, is a more or less pure toxemia, characterized by slowly progressing emaciation, marasmus, and finally death after complete exhaustion.

Symptoms in Man.—When persons are bitten by rabid dogs, if the wounds are not too lacerated, and if they are cleansed and treated in the proper antiseptic manner and burnt out with the actual cautery, they generally heal rapidly and do not give rise to any special local manifestations, except that there is occasionally a good deal of itching. There are no special symptoms during the period of incubation except that there may be more or less depression on account of apprehension. When the first symptoms of the stage of excitement are about to become manifest there may be a good deal of irritation at the site of the already healed wound, with lancinating pains shooting out from it; a slight tremor may be present in an extremity if it has been the seat of the bite. The voice becomes changed, it is not clear but slightly hoarse. The patient becomes decidedly depressed and irritable. Fever then generally occurs, and the disease progresses rapidly to its typical picture. The laryngeal symptoms become more severe, there is difficulty in respiration, the face becomes drawn, and shows an expression of great anxiety. Intense thirst compels the patient to make strong efforts at drinking, and these bring about contraction of the muscles of the larynx, so-called spasm of the glottis, with great air hunger or dyspnea. Salivation becomes marked and all reflexes are accentuated, so that a slight irritation may bring about attacks of muscular contractions. The patient, while very thirsty, is afraid to take water, and the mere thought of it may bring about spasm of the muscles of deglutition and of the larynx. As the disease progresses farther the convulsions affect more and more muscles, including the respiratory muscles of the thorax. Patients now often suffer from hallucinations and delirium, and

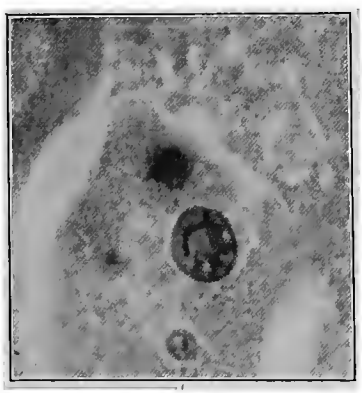
maniacal excitement may set in. This stage of excitement lasts from one to a few days, and then the paralytic stage sets in; the patient loses consciousness, goes down rapidly, and dies. The stage of excitement in man, as in dogs, may be very short, not well marked at all, and then the paralytic state develops almost immediately after the slight prodromal symptoms; this form of the disease is known as paralytic rabies or dumb rabies in dogs. Death in man from rabies generally occurs two to five days after the outbreak, or the terrible struggle may be prolonged to eight to ten days.

Pathologic Lesions.—There are no characteristic anatomic lesions from which a diagnosis of rabies in dogs or other animals could be made solely upon postmortem examination without the proper microscopic examination. Dogs dead from rabies generally show the paralysis of the muscles of the lower jaw, which hangs down and is not firmly closed as is generally the case in consequence of rigor mortis. The stomach in dogs and other carnivora frequently contains instead of remnants of food, undigestible foreign substances, such as wood, coal, pebbles, hay, straw, leather, etc. The mucosa is strongly congested, swollen, sometimes superficially ulcerated and hemorrhagic at the free margin of the folds. Foreign bodies are sometimes found in the esophagus or in the intestines. However, the presence of foreign bodies in the gastro-intestinal tract varies and their absence does not at all exclude a diagnosis of rabies. Mortley Axe made postmortem examinations of 200 rabid dogs. He found foreign bodies in 90 per cent. of the cases, but Galtier, in 1304 autopsies on rabid dogs found foreign bodies in the stomach in only 657 cases. The bladder is usually empty or contains a small amount of urine only, which generally contains glucose. Sometimes the bronchial mucosa is hyperemic, and the salivary glands are frequently swollen. The internal organs, such as the liver, spleen, and kidneys, are congested and sometimes show evidences of parenchymatous degeneration. The cerebrospinal meninges are edematous and hyperemic; the gray matter on section shows many bleeding points, indicating its congestion. Hart states that he has seen quite a number of cases of rabies in dogs where the postmortem examination showed the presence of food in the stomach and a normal mucous membrane.

Diagnosis.—*Changes in Nerve Ganglia.*—For a number of years attempts have been made to find changes in the central nervous system that would give a reliable diagnosis of rabies. Babes was the first to describe certain characteristic lesions. Schaffer, in 6 cases of hydrophobia in man, found in that part of the spinal cord where the nerves arise which go to the region where infection took place, also in the anterior gray horns, around the central canal, along the neuroglia bands of the white matter, in the perivascular lymph channels and in the vessel walls themselves, inflammatory round-cell infiltration and hemorrhages, both large and capillary. He also found in these regions hyaline degeneration of ganglion cells and vacuo-

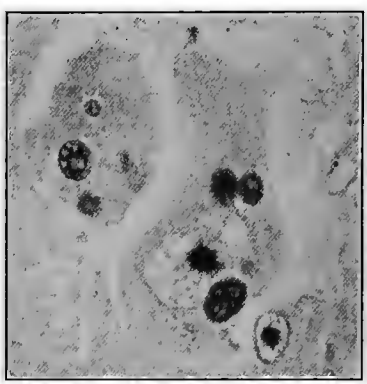
lation. A more important observation, which for a time gained great importance in the microscopic diagnosis of rabies, was made by Van Gehuchten and Nelis. They found in the cerebrospinal and sympathetic ganglia, particularly in the plexus nodosus of the pneumogastric nerve and in the upper cervical ganglia of the sympathetic nerve, a proliferation of the endothelial cells of the capsules of the ganglia. These proliferating cells invade the nerve cells of the ganglion, destroy them more or less, and may take their place entirely. The proliferating cells also infiltrate the perigangliomic region. The observations of Van Gehuchten and Nelis were soon confirmed by a number of observers, among others by Frothingham, who considers these findings as fairly accurate means to the diagnosis of rabies. He always found them present in cases of rabies and also in one case, that of a dog, where inoculations of emulsified central nervous material failed to produce rabies in the injected animals.

FIG. 212



"Negri bodies in nerve cells." × 2000. (After Wolbach.)

FIG. 213

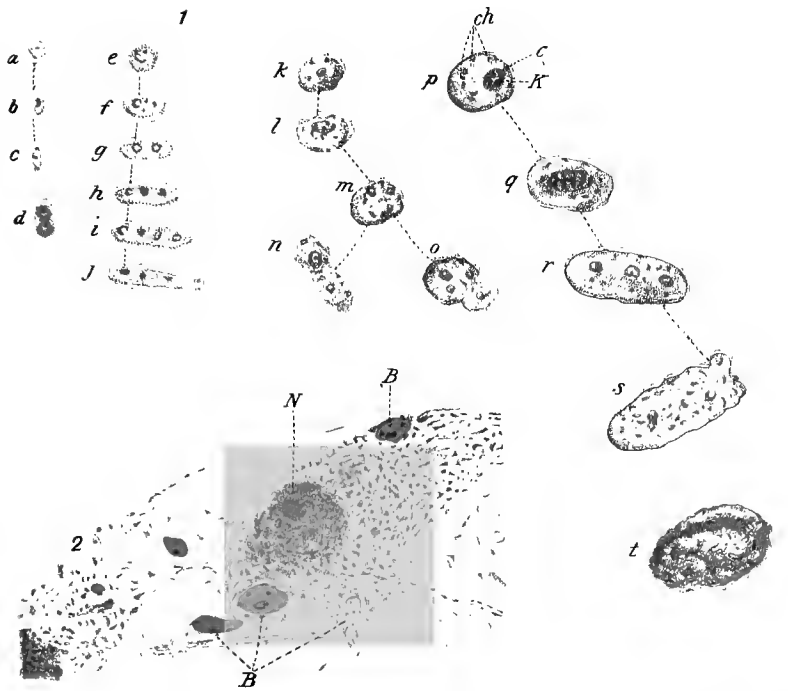


"Negri bodies in nerve cells." × 1000. (After Wolbach.)

Today the microscopic diagnosis of rabies is made upon the finding of the so-called

Negri Bodies.—Negri, in 1903, described certain inclusions in the ganglion cells of the central nervous system. He claimed that they were protozoa and the cause of rabies and the most reliable means of diagnosticating this disease in dogs, other animals, and man. These cell inclusions, now universally known as the Negri bodies, are contained in the protoplasm, particularly of the large ganglion cells of certain regions of the brain. They vary from minute dots to large bodies of 25 micra in diameter. Negri's observations were soon confirmed by numerous observers, and it can be stated today that these bodies are found almost without exception in all cases of natural rabies infection, so-called street rabies. However, after the virus has been passed a number of times through rabbits by subdural

PLATE XIV



1. Various division forms of the negri bodies (Giemsa stain). 2. Smear of Ammon's horn of dog, showing negri bodies (*B*) stained red in the large blue-stained nerve cells; *N*, nucleus of nerve cell. (Van Gieson's fuchsin and methylene-blue stain.) (Park.)

inoculation, that is, when the virus has become of the fixed type, only the smallest bodies are seen. The classification of these bodies, to which Williams has given the zoölogical name *neuroryctes hydrophobiæ*, cannot yet be regarded as established, and Calkins states that we are not yet justified in classifying them as sporozoa, because their variable form, their uninucleate condition, leading to chromatin distribution and budding, though found in other protozoa, are not characteristic for sporozoa. Williams and Lowden, whose careful researches of the Negri bodies have contributed much to our knowledge, describe them as follows: "They measure in size from 0.5

FIG. 214

"Negri bodies," or Neuroryctes hydrophobiæ, in different stages of chromatin distribution. (After Negri.)

to 18 micra (Negri saw some as large as 25 micra); no very large forms are found in the early stages of the disease, and in the fixed virus infection only the very small forms are found. They vary much in shape, from mere irregular points to larger, round, oval, or elongated bodies. They are generally inside of the large ganglion cells, but in smear preparations (see below) they may be found squeezed out of the cells. Whatever the variety of species of animals infected the bodies preserve their same general characteristic structure, namely, a hyaline cytoplasm with an entire margin, and with one or

more inner bodies having a more or less complicated and regular structure." Negri early recognized that the rabies bodies in the ganglion cells of the brain had a nucleus, and he later stated that their chromatin had either a solid or a reticular structure, while their cytoplasm contained a variable number of chromatin granules. The latter are frequently arranged, as stated by Williams and Lowden, in a more or less complete circle about the nucleus. They are somewhat irregular in outline and size, being occasionally ring-shaped, sometimes elongated, often in twos, due probably to the ative changes of growth and division. The difference in shape of the Negri bodies, such as round, triangular, etc., is due to their position in the ganglion cells, since their bodies are very plastic and easily adaptable to a variety of positions. The variability in shape is also probably largely due to a rapid multiplication. The division forms suggest rapid growth and multiplication. The elongated forms, containing from two to five or even six nuclei, are the result of rapid nuclear division without corresponding cell division. This condition is found quite frequently in protozoa. The elongation of the protoplasm is probably due to the position of these bodies between the nerve fibrils, and to their great plasticity.

Under the most favorable conditions (fixed virus), growth and division occur most rapidly and simply, the tiny forms dividing and redividing apparently indefinitely. Small mulberry masses are found during this stage, but whether they are the result of the breaking up of a larger form or of the rapid division of a tiny form it is impossible as yet to say.

In cases where there has been an inoculation of comparatively small quantities of the virus, *i. e.*, a small number of forms of the parasite capable of immediate infection, or in cases where there has been an infection of less susceptible animals (dogs, cattle, human beings, etc.), or with a less accustomed virus (fixed virus of rabbits into guinea-pigs or mice), a slower growth is obtained with its larger structures and different division forms. The chromatin accumulation in the form of a definite nucleus apparently undergoes fragmentation very easily, resulting in forms containing two to several central bodies, some rounded, some elongated, some of unequal division similar to budding. Then forms are found with bodies apparently differentiated within one membrane, and bodies with practically all stages of hour-glass constriction, indicating transverse division. Many pairs, unequal in size, apparently fusing or dividing, have been seen, and finally, large bodies with the chromatin scattered throughout the whole organism in the form of tiny, unevenly rounded, or elongated masses. One or two will be larger, indicating the remains of the nucleus. In these forms are found all stages of apparent budding, varying somewhat in size, some being very tiny. The formation of buds accounts for the appearance in the same cell of both large and small forms. It also helps to account for the rapid

spread of the organisms. These tiny budded forms similar to "swarm spores" are probably motile and pass quickly to other host cells.

The Rapid Microscopic Diagnosis of Rabies.—Williams and Lowden worked out a rapid method of diagnosticating microscopically rabies, and an almost identical method by Frothingham was published about the same time.

The author has used their smear method since the fall of 1906 and has found it very satisfactory and reliable, and it is highly recommended in all cases where a rapid microscopic diagnosis is necessary. The steps of Williams' and Lowden's method are as follows:

1. Glass slides and cover-glasses are washed thoroughly with soap and water, then heated in the flame to get rid of greasy substance.

2. A small bit of the gray substance of the brain to be examined is cut out with a small, sharp pair of scissors and placed on the slide about one inch from the end, so as to leave enough room for a label. The cut in the brain should be made at right angles to its surface and a thin slice taken, avoiding the white matter as much as possible.

3. A cover-slip placed over the piece of tissue is pressed upon it until the brain substance is spread out in a moderately thin layer, then the cover-slip is moved slowly and evenly over the slide to the end opposite the label. Only slight pressure should be used in making the smear, but slightly more should be exerted on the cover-glass toward the label side of the slide, thus allowing more of the nerve tissue to be carried farther down the slide and producing better spread nerve cells. If any thick places are left at the edge of the smear, one or two of them may be spread out toward the side of the slide with the edge of the cover-glass. If the first smear does not turn out successfully others should be made until a satisfactory one is obtained.

4. For diagnosis work such a smear should be made from at least *three different parts* of gray matter of the central nervous system: First, from the cortex in the region of the *fissure of Rolando* or in the region corresponding to it (in the dog the convolution around the crucial sulcus); second, *from Ammon's horn;* third, from the *cerebellum.*

5. The smears are dried in air and subjected to one or both of the following staining methods:

(*a*) *Giemsa's Solution.*—The smears are fixed in methyl alcohol (commercial is just as good as pure) for about five minutes. The staining solution recommended last by Giemsa is as follows: 1 drop of the stain to every c.c. of distilled water made alkaline by the previous addition of 1 drop of a 1 per cent. solution of potassium carbonate to 10 c.c. of water. This is poured over the slide and allowed to stand from one-half to three hours. The longer time brings out the structure better, and in twenty-four hours well-made smears are not overstained. After the stain is poured off the smear is washed in running tap water for one to three minutes, and dried

with filter paper. If the smear is thick the "bodies" may come out a little more clearly by dipping in 50 per cent. methyl alcohol before washing in water, then the washing need not be as thorough. By this method of staining the cytoplasm of the "bodies" stains blue and the central bodies and chromatoid granules stain a blue red or azure. Generally the larger "bodies" are a darker blue than the smaller; the smallest of all may be very light. The stain varies somewhat according to the thickness of the smear. Some have a robin's egg blue tint, but this is due to long fixation in the methyl alcohol. In this case the red blood cells may have a greenish tint. The cytoplasm of the nerve cells stains blue also, but with a successfully made smear the cytoplasm is so spread out that the outline and structure of most of the "bodies" are seen distinctly within it. The nuclei of the nerve cells are stained red with the azure, the nucleoli a dull blue, the red blood cells a pink yellow, more pink if the decolorization is used. The "bodies" have an appearance of depth, due to their slightly refractive qualities.

For diagnostic purposes this method of staining may be shortened as follows: Methyl alcohol, five minutes, equal parts of the Giemsa solution and distilled water, ten minutes. In this way "bodies" are generally brought out well enough for diagnosis, and sometimes the structure shows distinctly. It is always well, however, to make smears enough for the longer method of staining, in case the shorter should prove unsatisfactory.

(*b*) *The eosin-methylene blue method* recommended by Mallory. The smears are fixed in Zenker's solution for one-half hour; after being rinsed in tap water they are placed successively in 95 per cent. alcohol + iodin, one-quarter hour; 95 per cent. alcohol, one-half hour; absolute alcohol, one-half hour; saturated watery eosin solution, twenty minutes, rinsed in tap water; alkaline methylene-blue solution, fifteen minutes; differentiated in 95 per cent. alcohol lasting from one to five minutes, and dried with filter paper. With this method of staining the cytoplasm of the "bodies" is a magenta, light in the small bodies, darker in the larger; the central bodies and chromatoid granules are very dark blue, the nerve cytoplasm a light blue, the nucleus a darker blue, and the red blood cells a brilliant eosin pink. With more decolorization in the alcohol the "bodies" are not such a deep magenta and the difference in color between them and the red blood cells is not so marked.

The "bodies" and the structure are often more clearly defined with this method, and perhaps on the whole it is better to use it for making diagnoses; but when there are only tiny "bodies" present, or when the brain tissue is old and soft, the Giemsa stain seems to be the more successful. Above all, when one wishes to study the nature of the central structures and granules the Giemsa stain must be used. Both methods are strongly recommended.

The technique of the section work is as follows: (1) The small pieces are left in Zenker's fluid for three to four hours; (2) washed

in tap water for five minutes; (3) placed in 80 per cent. alcohol + iodin (enough tincture of iodin added to give port-wine color) for about twenty-four hours; (4) 95 per cent. alcohol + iodin, twenty-four hours; (5) 95 per cent. alcohol, twenty-four hours; (6) absolute alcohol, from four to six hours; (7) cedar oil until cleared; (8) cedar oil + paraffin, 52°, āā, two hours; (9) paraffin, 52°, two hours in each of two baths; (10) boxing; (11) sections are cut at 3 to 6 micra, dried in thermostat at 36° C. for about twenty-four hours, protected from the dust, and stained according to the eosin-methylene-blue method of Mallory. The important point in the technique is the time the material is allowed to remain in Zenker. According to the author's experience two hours' fixation is not enough, three or four hours is very good, and with every hour after five hours the result becomes less satisfactory. Left in Zenker overnight the tissue is granular and takes the eosin stain more or less deeply, both of which results interfere with the appearance of the tiniest "bodies," especially of the very delicate, tiny forms found in sections from fixed virus. Another point in favor of the short fixation in Zenker is that the precipitate formed by the mercury is not so great, and is more easily eliminated, which is a very great help in the identification of the tiniest forms.

For routine work for diagnostic purposes the method of fixing the smears in Zenker's solution and staining subsequently by the eosin-methylene-blue method of Mallory is the simplest and most reliable.

Methods of Preparing Material for Laboratory Examination.—Few veterinarians are so situated and trained that they can make a dependable microscopic diagnosis of rabies in a dog. However, if a suspected animal has bitten other dogs or cattle, horses, persons, etc., a definite diagnosis should always be made, so that the proper measures can be taken if the animal did suffer from rabies. One of the worst things to do if a suspicious dog has bitten a person is to kill the dog at once, because in the very earliest stages of rabies it may be impossible to find the Negri bodies, yet the dog may have had rabies and its saliva may already have been infective. The proper thing to do is to isolate and safely detain such a dog and to watch for the development of symptoms. If the animal sickens under typical symptoms of rabies it may be killed and the brain examined microscopically, or if the symptoms are not clear it may be allowed to die and then the brain should be examined. The veterinarian in charge of the case should make a postmortem examination on the dog and the head should be severed from the body somewhere in the middle of the cervical vertebræ, so that the upper cervical ganglia are left undisturbed, that they may, if desirable, be included in the microscopic examination. The head should then be wrapped in a piece of cheese cloth, or, if available, in a thin rubber sheet, as used in surgical dressings, placed in a tin bucket, then in a wooden box, and forwarded by express

to the laboratory where the examination is to be made. During the warm season the tin bucket should be surrounded by cracked ice. These measures, however, are sufficient only when the package can reach its destination, particularly in summer, in less than twenty-four hours. If it has to be on the road longer, or if the weather is very warm, it is better to take the brain out of the cranium. The entire brain can then be placed in pure neutral glycerin and sent to the laboratory. This method has been frequently recommended, but it is almost impossible to find the Negri bodies in a brain that has been preserved in glycerin. The following method is, therefore, better: Take out the brain, divide into two equal halves. Place one half into several times its bulk of pure neutral glycerin, the other half into a strong formalin solution (1 part of formalin to 4 parts of water). When the brain arrives at the laboratory, very small pieces, not more than 1 square centimeter large and less than half a centimeter thick, are cut out from the gray matter of the Ammon's horn, the region of the fissure of Rolando and the cortex of the cerebellum of the formalin preserved material. These pieces are placed in Zenker's solution for a few hours, then washed rapidly in running water, then placed for one hour in pure water-free acetone, which is changed once, and then dropped into melted paraffin, sectioned, and stained by the eosin-methylene-blue method. The fixation by Zenker's fluid may even be left out and the sections can then be simply stained by hematoxylin and eosin. The watery eosin solution should then be somewhat stronger than when used for ordinary work in histology or pathology. If the Negri bodies are found the other half of the brain in glycerin is not needed, but if they are not found and the investigation is to be carried on, an emulsion in physiologic salt solution is made from the brain substance in glycerin and injected into rabbits by the subdural method. This necessitates opening the cranium by trephining, followed by the subdural injection. (Details of this method of inoculation are given below.)

Are the Negri Bodies Protozoa?—That the Negri bodies are indeed protozoa is not accepted as an established fact by all observers, and some hold that they are degenerative or other products of elements of the central nervous system of the animal or person infected with rabies. The main reason for this belief is the observation first made by Remlinger and Riffat-Bey, and confirmed by others, that emulsions of the central nervous system frequently can be filtered through Chamberland and Berkefeld filters without losing their infectiousness. However, it must not be forgotten that there are very small Negri bodies which are probably very plastic and which under pressure may easily pass the pores of porcelain or clay filters. The preponderance of evidence today appears to be in favor of the protozoan nature of the Negri bodies.

Peculiar Biological Properties of Neuroryctes Hydrophobiæ.—If the Negri bodies are protozoa, strict parasites which live and multiply in

the ganglion cells of the central nervous systems of higher animals, they furnish one of the most wonderful examples of how a parasite in the struggle for existence, with the survival of the fittest, may develop those properties which will insure its propagation from one host to another. To the parasitic tubercle bacillus are open a thousand and one routes by which it may be transferred from one animal to another; to the neuroryctes hydrophobiæ, as far as known, only one way, namely, that of an infected animal biting and wounding another. There is no disease which, particularly in carnivora, leads to such an intense, insane desire to bite as does hydrophobia, and this symptom must be looked upon as one due to disturbances in the ganglion cells depending upon poisons developed and produced by the neuroryctes for the sole and special purpose of insuring the survival of the race after the death of the host, which it kills by its own multiplication. That the parasitic microörganisms of rabies should produce as one of the very prominent symptoms of the disease the psychic disturbances which lead to the intense desire to bite, particularly in canines, cats, rats, and occasionally in rabbits, is the more remarkable when it is considered that the tetanus toxin which also directly affects the central nervous system travels to it along the peripheral nerves, produces symptoms, as far as convulsions and paralyses are concerned, very similar to the rabic virus, does not in any way lead to similar or identical psychic disturbances. Tetanus, however, cannot be spread through the bite of an infected animal and the generally saprophytic tetanus bacillus does not depend for the preservation of the race upon the invasion of higher animals.

Another striking feature in the life history of neuroryctes hydrophobiæ is the fact that while not to any extent found in other tissues but those of the central nervous system, it is always present in the salivary glands and excreted with the saliva to thus insure its transfer to a new host. The mode of transfer from one host to another is identical in principle with that found in malaria. However, in this disease the protozoan parasites are first taken up by an intermediary host, the mosquito, to wander from its stomach into the salivary glands, and to be transferred by biting to its more permanent host, man, birds, or other animals.

Spread of the Virus in the Organism of Susceptible Animals.—It has been shown by the experiments of Pasteur and others that the rabic virus from its place of entrance in the body of a susceptible animal travels along the peripheral nerves to the central nervous system. Rabies can always be produced by injecting the virus directly into the peripheral nerves or even by moistening with an emulsion the central end of a divided nerve. On the other hand, if the peripheral end of a cut nerve is infected and the connection between this part and the central nervous system carefully destroyed then infection does not occur. The most pronounced changes in the cord in rabies are always found in that part which supplies the peripheral nerves

to the region where the infection by bites occurred. Hellman and Marx have shown that if virus is carefully injected into the peritoneal cavity in such a manner that nerves are not injured, infection does not occur. Intravenous injection leads to infection in dogs and rabbits but not in herbivora, which shows that the possibility of infection through the blood current cannot be denied, but everything points to the peripheral nerves as the common routes by which the virus usually travels to the central nervous system.

Immunization in Rabies.—The first attempts to immunize animals were made in 1881 by Gaultier, who injected saliva from rabid dogs into the jugular veins of a number of sheep and one horse. This procedure did not produce rabies in the animals so treated, and, according to the claim of the experimenter, protected them against subsequent bites from hydrophobic dogs. Nocard and Roux repeated Gaultier's experiments on sheep, goats, cattle, and horses, but instead of using saliva they employed for the intravenous injections emulsions from the central nervous system, and they confirmed the observation of their predecessor, that such treatment produced immunity against subsequent intraocular inoculation and against the bites of rabid animals.

The Pasteur Treatment.—Between the experiments of Gaultier and those of Nocard and Roux, Pasteur had taken up the work of immunization against rabies. He had previously discovered the method to immunize animals against fowl cholera, hog erysipelas, and anthrax by the use of attenuated cultures or viruses, and he based his experiments on rabies from the start upon attempts to prepare an attenuated virus. He first succeeded in obtaining it by repeated passages through monkeys. If a virus so obtained was injected subcutaneously into dogs it did not produce hydrophobia and protected the animals so treated against the subsequent bites of rabid dogs. The method on which the so-called Pasteur treatment of hydrophobia is based was subsequently worked out by Pasteur, Chamberland, and Roux. Its principle is the following: Rabbits are first inoculated subdurally from the virus obtained from dogs which have developed rabies. This is the so-called *street virus*. It is of variable virulency and produces hydrophobia in rabbits after a variable period of incubation. If the virus is then passed on from rabbit to rabbit, always by *subdural inoculation*, the period of incubation is more and more shortened on account of the increasing virulency of the living poison. After a number of passages the virulency reaches a certain maximum beyond which it cannot be increased, and the period of incubation becomes stationary at six to seven days. The virus so obtained, which also shows an increased virulency when inoculated subdurally into animals other than rabbits, is known as the *fixed virus*. It can be attenuated by a variety of methods, but the method first employed by Pasteur and still used by many consists in exposing the spinal cords of rabbits which contain the fixed virus to drying-out processes.

The longer the drying-out process is allowed to go on the more attenuated becomes the virus.

The emulsion used in inoculating rabbits and also in the protective inoculations is always prepared from the cords of rabbits containing the fixed virus by rubbing up pieces of the cord with sterile physiological salt solution until a milky emulsion is obtained. It is, of course, always necessary first to start from the cord of a hydrophobic dog and to continue inoculating rabbits subdurally until a fixed virus has been obtained.

Technique.—The technique of subdural inoculation in rabbits is as follows: The animal is fastened to a small animal operating table, resting on its abdomen, and the head is fixed so that it cannot move.[1] The scalp is then cut open very near the median line from near the posterior angle of the eye toward the insertion of the ear. The periosteum is next removed from the bone. The latter is then perforated by a small trephine, the tip of which is not more than about 2 to 3 mm. in diameter. The piece of bone cut out is removed by a small hook. The dura mater then becomes visible and it is perforated by the fine needle of a hypodermic syringe and from $\frac{1}{8}$ to $\frac{1}{4}$ c.c. of the emulsion is slowly injected into the subdural space. In inserting the needle it should be held rather obliquely and directed forward and outward; in this manner injury to the brain is avoided. After the emulsion has been injected and the needle withdrawn the linear incision of the scalp is closed by sutures and a collodion dressing is put on the wound. It goes without saying that everything has to be done aseptically in order to avoid the occurrence of a septic meningitis which might kill the experimental animal before rabies had time to develop. This generally occurs nine to twenty-one days after the inoculation of the street virus and six to seven days after the inoculation of the fixed virus. The subdural method is used exclusively in the preparation of the fixed virus; it is the only reliable one, and it alone should be used in diagnostic work. Even the intraocular method is not absolutely reliable, and all other methods are very unreliable. Rabbits inoculated with street virus generally develop the picture of dumb rabies; however, this is not invariably the case, and they sometimes develop the furious type, become aggressive, and are liable to bite. Since the cases where hydrophobic rabbits did bite persons all occurred in laboratories, and since the persons bitten all promptly received treatment, it is not known whether such an injury might lead to an outbreak of hydrophobia.

Rabbits after being inoculated with street virus, before developing typical symptoms, generally show, as first noticed by Babes, an elevation of temperature. Others have denied that this prodromal elevation of temperature regularly occurs. This is followed by a

[1] Kraus states that it is not necessary to tie rabbits to the operating table; they can be held by an assistant and the operation can be performed. This saves much time if a number of animals are to be inoculated.

slight paralysis of the hind legs, so that the animals can be easily thrown over. They then become restless and cramps of the muscles of the lower jaw occur. The disease now may take on the furious type, but more generally the paralyses become more marked, the hind legs are completely paralyzed, and the front legs become likewise affected. Within a few days great emaciation occurs and death generally follows four to five days after the first symptoms became manifest. After subdural inoculation of the fixed virus the furious type of rabies is never developed in rabbits.

Preparation of the Attenuated Virus.—Where material for the Pasteur treatment of rabies is prepared it is necessary to inoculate at least two rabbits every day, so that a complete series of attenuated viruses is always on hand, even if occasionally one cord should become spoiled by bacterial contamination. The inoculated animals must be kept separate from the others, and they must be carefully watched for the development of any other disease, which would make them unavailable for use for the preparation of rabies virus. The inoculated animals are killed by cutting the throat and bleeding, about twenty-four hours before their expected death from rabies. They are then skinned, the abdominal and thoracic cavities are opened and examined. If no other pathologic lesions but those of rabies are seen the animals are stretched out on a sterile board, abdomen down, and the external surface of the back is thoroughly washed with a solution of lysol. The muscles of the back are removed from the vertebral column and the latter is then opened with special scissors devised for the purpose; next the roots of the spinal nerves on each side are severed with a fine knife, the dura mater is laid open, and the upper part of the cord is tied with a piece of sterile silk or grasped with a small, sterile platinum hook. The cord is then lifted out and cut in the middle of its course. The upper piece is at once placed in a wide-mouthed sterile flask and so suspended that it does not touch the walls of the vessel. Next a small portion of the lower part is cut off and dropped into a sterile Petri dish or other sterile dish for subsequent examination, as to the absence of contamination by bacteria. Then the small lower portion is removed and treated like the upper portion. The bottles in which the pieces of cord are suspended are of special construction and contain caustic soda which is used to absorb water and bring about the drying out of the material. The flasks with the pieces of cord are kept in a perfectly dark place where the temperature is stationary at 20° C. After the cord has been taken care of the internal organs of the abdominal and thoracic cavities and the brain are carefully dissected to show that they are free from pathologic lesions which would bar the material from use. When the pieces of cord have gone through the drying-out process they are rubbed up and emulsified with sterile bouillon, or, still better, with Babes' fluid, consisting of sulphate of sodium, 5 gr.; chloride of sodium, 6 gr., and water enough to make

1000 c.c. One gram of cord is rubbed up with 5 c.c. of fluid. Of this emulsion 1 to 3 c.c. are used on persons which receive the preventive Pasteur treatment, the dose varying as to the severity of the case and as to the age of the patient. Pasteur devised three methods which are still practised: one for cases of light wounds which came to treatment immediately, the two others for more severe cases and for later treatments. In all cases treatment is begun with an emulsion from a cord which has been subjected to the drying-out process for fourteen days, next a cord is used which has been dried out for thirteen days, and so on, until finally a cord is used which has been kept in a bottle over caustic soda for three days. The whole course of treatment lasts from eighteen to twenty-one days, because the same type of emulsion is used on two consecutive days at times. Wounds of the face are always treated by the most energetic method, which is carried out for twenty-one days.

The original method of Pasteur has been modified by a number of authors. Hoegys uses fresh virus obtained from the medulla of the rabbit. The fully virulent tissue is first emulsified with sterile physiologic salt solution in the proportion of 1 to 100. Then the following dilutions are prepared: 1 to 200, 1 to 500, 1 to 1000, 1 to 2000, 1 to 5000, 1 to 10,000. Three c.c. of the last-named dilution forms the first injection, and finally 1 c.c. of the strongest vaccine; *i. e.*, 1 to 100 is given on the twentieth day of the treatment. Babes uses a fixed virus which has been attenuated by being heated to 80° C. Tizzoni and Catanni use a virus attenuated by artificial gastric juice.

There have now been treated by Pasteur's original method or one of its modifications many thousand persons bitten by rabid animals, and the mortality among those treated is less than 1 per cent. This result shows clearly the great efficiency of the method. It is believed that the results will still be improved by using for the vaccination less attenuated and more virulent fixed virus, because it appears from the work of Marx, Babes, and Nitch that the fixed virus of the rabbit for man in subcutaneous injection represents already an attenuated virus. Kraus believes that the best method of inoculation after bites of rabid animals, particularly after extensive lacerations, will be the subcutaneous injection of comparatively large doses of the virulent fixed virus.

Cord Lesions after the Pasteur Treatment.—The Pasteur treatment for rabies does not, in the vast majority of cases, lead to any disturbances due to the injections themselves. However, there have been reported a number of cases—less than 1 for each 1000 cases treated—in which the injections appear to have led to some transitory lesions in the cords of the persons treated. These lesions manifest themselves by disturbances of sensation, disturbance of the reflexes, slight paresis or paralysis and the symptoms developed sometimes resemble somewhat the very serious disease known as Landry's paralysis. That these symptoms are not an abortive form of rabies, but due in some

way to the injection of the fixed virus, is shown by the fact that they have occurred in persons who, as shown subsequently, were not bitten by rabid dogs, but who received the Pasteur treatment. Almost all of these cases end in complete recovery. There is, however, one case reported, that of a man sixty-two years old, who died, but it is doubtful whether he died in consequence of the Pasteur treatment or from some other cause.

Differences in Virulency between Street and Fixed Viruses.—The fixed virus, while of a maximum virulency subdurally for rabbits and other animals, if injected by any other route, exhibits a decreased virulency and can be used for immunizing purposes. Marx, discussing the differences between street and fixed virus, comes to the following conclusions: The fixed virus produces a more abundant or a more powerful toxin than the street virus. The rate of velocity of multiplication of the fixed virus is greater than that of the street virus; the fixed virus in purely subcutaneous inoculation is absolutely harmless for man and apparently much less virulent for animals than the street virus. For some animals it is also less virulent in intramuscular (monkey) and in intraocular (monkey, rabbit) inoculation. This behavior can only be explained—everything being equal—by assuming that the fixed virus is more easily destroyed by the protective powers of the body than the street virus.

The question then naturally arises, Why should the stronger, more rapidly multiplying, fixed virus be more easily destroyed by the protective powers of the body? Marx and others, discussing this point, do not offer any explanation. The following, however, suggests itself to the author:

In natural infection the virus, in order to produce rabies, must be able to wander in some way from the portal of entrance, mainly along the peripheral nerves to the central nervous system, there to invade the ganglion cells, to multiply and to produce the specific disease hydrophobia. By injecting the street virus for a number of generations subdurally, a method of procedure which, of course, is highly unnatural, we artificially breed an abnormal race of microorganisms. These become more highly specialized in their parasitism and lose their organs of locomotion or other apparatus, which enabled them to travel from a portal of entrance in the subcutaneous connective tissue to the central nervous system. The statement about the loss of organs of locomotion is, of course, not to be taken literally, but simply in the same sense as Ehrlich has depicted as definite geometrical bodies, antigens and antibodies, amboceptors, complements, etc., in order to give a clear idea of their combination neutralization, fixation, lysis, agglutination, etc.

If the microörganisms of rabies in consequence of continued subdural injection have lost the power to travel from any outside place toward the central nervous system, they will be destroyed in loco by the protective powers of the invaded organism, and while this

occurs antibodies will be formed which prevent the organisms of the street virus from producing an attack of hydrophobia. That there is, indeed, a morphologic difference between the street virus and the fixed virus is shown by the fact that the latter never produces the large Negri bodies but only the smallest forms of them.

Resistance.—The rabies virus possesses a moderate resistance. However, it must not be forgotten that the action of antiseptics, etc., cannot be studied upon the pure virus, as it is always more or less protected by the natural substances or body fluids in which it is contained. In a tabulation by Heim, compiled from the work of different authors, the following figures are given: The rabies virus is destroyed by 1 to 1000 bichloride of mercury in two to three hours; 1 per cent. carbolic acid in two to three hours; 5 per cent. carbolic acid, 5 per cent. salicylic acid, 10 per cent. sulphate of copper, 1 per cent. kreolin in five minutes; 70 per cent alcohol in twenty-four hours. Formalin vapors fifteen to forty-five minutes; gastric juice after twenty-four hours; exposure to 45° C. in twenty-four hours; 50° C. in one hour; 52° to 58° C. one-half hour; 60° C. very rapidly. Low temperatures have no effect upon the virus. Frothingham found the cord of a rabid dog kept at —40° C. virulent after one year and ten months. The virus remains virulent longer in buried cadavers than in those exposed to the air and light. *Glycerin does not destroy the virus, in fact, it acts as a conservative for it for months.*

Antirabic Serum.—Babes and Lepp were the first to show, as early as 1889, that the serum of dogs immunized by Pasteur's method contained substances which are antagonistic to rabies virus, and, therefore, can be used to neutralize it, and also, as is claimed, be employed to protect dogs by passive immunization against inoculation with fixed virus and against the bites of dogs suffering from hydrophobia. These early experiments of Babes and Lepp appear to have been the first to demonstrate the formation of antibodies against a virus, and of the employment of such antitoxin to produce what is now universally known as passive immunization. However, the claim that antirabic serum can cure hydrophobia after it has made its appearance or can favorably modify its course is not admitted as correct by Kraus. Babes and those who have used an antirabic serum on man bitten by rabid animals have never used it alone but always in combination with fixed virus. In other words, they have used the simultaneous method, never the method of pure passive immunization, and it is claimed that the results of the simultaneous method have not been better than the uncombined treatment with fixed virus. The antirabic serum of Babes is prepared by first treating rabbits, donkeys, dogs, and sheep by Pasteur's method and subsequently injecting increasing, finally large, doses of fixed virus. The blood of the hyperimmunized animals is drawn ten to twenty-five days after the last injection of virus and the blood serum is obtained in the same manner as in the case of diphtheria or tetanus antitoxin

preparation. The immune serum is standardized, according to Kraus, with a fixed virus prepared fresh from the medulla of a rabbit in the proportion of 1 part emulsified with 100 parts of physiologic salt solution and filtered through a paper filter to remove the remaining coarse particles. Antiserum and virus are mixed in varying proportions and are allowed to stand for twenty-four hours at room temperature. The mixtures are then injected subdurally into various animals. While Kraus, like Babes and others, succeeded in destroying the fixed virus by mixing it with antiserum, he never succeeded in obtaining any protective influence by injecting the antiserum even in large doses into animals which were subsequently infected with rabies.

QUESTIONS.

1. What are the other scientific and common names for rabies?
2. How long has the disease been known? Where does it occur?
3. How is it generally spread in natural infection?
4. When does the saliva of a dog suffering from rabies become infective?
5. What animals are susceptible to rabies?
6. Discuss the period of incubation of rabies. May it be prolonged to one or more years?
7. What explanation suggests itself as to such a long period of incubation?
8. What percentage of people bitten by rabid dogs develop rabies? What percentage of dogs bitten by other dogs?
9. What is probably the behavior of the rabies virus after its entrance into the body of persons and animals?
10. What are the different forms of rabies?
11. Describe the symptoms of furious rabies in a dog. Also of dumb rabies.
12. What is the consumptive form of rabies?
13. Does recovery from rabies in dogs occur?
14. Describe the symptoms of rabies in man.
15. Describe the postmortem findings on a dog dead from rabies.
16. Describe the changes of Van Gehuchten and Nelis in the cervical and spinal ganglia in rabies.
17. What are the Negri bodies? In what parts of the central nervous system would you look for them?
18. Describe their morphology in the brain of a dog dead from rabies.
19. What is the meaning of street virus and of fixed virus?
20. What is the difference between the Negri bodies in street and in fixed virus cases?
21. Are the Negri bodies found in the earliest stages of rabies? If not, what practical measures should be adopted in the diagnosis of rabies by the aid of the Negri bodies?
22. Give in detail the steps in the rapid microscopic diagnosis of rabies in the dog.
23. Describe the best fixing and staining methods used in this rapid diagnosis.
24. How should material from a suspected rabid dog be prepared for subsequent laboratory examinations?
25. What are the opinions as to the nature of the Negri bodies?
26. What are the peculiar biologic properties of the Negri bodies which enable them to survive as a race of intracellular parasites?
27. How does the virus of rabies spread in the body of susceptible animals from its portal of entrance to the central nervous system?
28. Describe the method to inoculate rabbits subdurally with the street virus
29. How is the fixed virus prepared from the street virus?
30. How long is the period of incubation of the fixed virus inoculated subdurally?
31. Is the fixed virus in subdural inoculation virulent for animals other than rabbits?

32. What is the principle of the Pasteur treatment of rabies?
33. How is the material for this treatment obtained, prepared, and used on persons?
34. What forms of rabies is seen in rabbits?
35. What is Babes' fluid? For what purpose used?
36. In what kinds of injuries is the most energetic Pasteur treatment to be used?
37. What other methods are used to attenuate the fixed virus?
38. What would be the effect of injecting a large dose of fixed virus subcutaneously into a person?
39. Does the Pasteur treatment sometimes lead to any disturbance due to the inoculations? If so, what kind of disturbances?
40. Discuss the differences in virulency between the street and the fixed viruses in subcutaneous inoculation.
41. What hypothesis have you to offer to explain these differences?
42. Discuss the resistance of the virus of rabies.
43. What is known about the preparation and effect of an antirabic (immune) serum?
44. What effective method is there to protect an animal by passive immunization against rabies? What is the curative value of the rabies immune serum?

APPENDIX.

Metric System.—The metric system, which is now universally employed in all scientific measurements, and also in every-day life in most European countries, is based upon the meter as the primary object of length. One meter is equal to $\frac{1}{10000000}$ part of the distance measured on a meridian of the earth from the equator to the pole and equals about 39.37 inches. The original meter is a platinum bar kept in the public archives of France and from this original standard other nations have procured copies. The metric system, based upon the meter, is a decimal system, *i. e.*, one in which all values are fractions in tenths or multiples of ten. The meter is first divided into 100 equal parts and $\frac{1}{100}$ meter is called a centimeter; each centimeter is subdivided into 10 parts and this length is called a millimeter. For microscopic measurement the millimeter is again subdivided into 1000 parts, and this unit is called 1 micromillimeter, one micron, or 1μ. The following are the most important subdivisions:

1 micromillimeter	=	0.001	millimeter
1 millimeter	=	0.001	meter
1 centimeter	=	0.01	meter
1 decimeter	=	0.1	meter
1 meter			
1 decameter	=	10	meters
1 hectometer	=	100	meters
1 kilometer	=	1000	meters
1 myriameter	=	10,000	meters

EQUIVALENTS IN INCHES.

1 micromillimeter	=	0.0000394	inch
1 millimeter	=	0.0394	inch
1 centimeter	=	0.3937	inch
1 meter	=	39.37	inches

The surface measures derived from the meter are:

1 square centimeter
1 square meter
100 square meters = 1 Are = 119.6 square yards
10,000 square meters = 1 Hectare = 2.471 acres

The cubic measures are:

1 cubic centimeter = 0.27 fluidrachm
10 cubic centimeters = 0.338 fluidounce
1000 cubic centimeters = 1 liter = 0.909 quart

One cubic centimeter of distilled water at 4° C. in the metric system has been taken as the unit of the system of weight. This mass of water, equal to 1 cubic centimeter (1 c.c.), is called *one gram,* and from it the following weights are derived:

1 milligram	=	0.001	gram	=	0.0154 grain (avoirdupois).
1 centigram	=	0.01	gram	=	0.1543 grain
1 decigram	=	0.1	gram.	=	1.5432 grain
1 gram	=			=	15.432 grains
1 kilogram	=	1000.	grams	=	2.2046 pounds.

The following table shows the equivalents in the metric system to some of the common weights and measures:

1 inch	=	2.54	centimeters
1 foot	=	0.3048	meter
1 yard	=	0.9244	meter
1 rod	=	5.029	meters
1 mile	=	1.0933	kilometers
1 square inch	=	6.452	square centimeters
1 square foot	=	0.0929	square meter
1 square yard	=	0.8361	square meter
1 acre	=	0.4047	hectare
1 square mile	=	259.	hectare
1 cubic inch	=	16.29	cubic centimeters
1 liquid quart	=	0.9465	liter
1 liquid gallon	=	3.786	liters
1 ounce avoirdupois	=	28.35	grams
1 pound avoirdupois	=	0.4536	kilogram
1 ounce Troy	=	31.104	grams
1 pound Troy	=	0.3732	kilogram

Thermometer Scales.—The principle on which thermometers are constructed is the following: A fine glass tube, having a closed bulb on its lower end, contains chemically pure mercury. The latter is heated in a boiling, strong, salt solution. The mercury then rises in the tube, evaporates partly, and expels all the air from the upper open end of the tube. If now this upper end is fused and the mercury allowed to cool a vacuum is formed above it, and when the mercury again expands it meets with no resistance. The thermometer tube, after having been sealed at the upper end, is placed in melting ice and the point to which the mercury column reaches is marked the freezing point of water (zero = 0). The thermometer is then immersed in boiling distilled water and the point to which the expanded mercury now reaches is marked as the boiling point of water. The space between the freezing point of water and its boiling point is marked off into a number of equal degrees. In the *Celsius* scale the space is marked off into 100 equal parts, but in the Réaumur scale into 80 equal parts. The thermometer scale most commonly in use in this country in everyday life originated in a somewhat ridiculous manner. Fahrenheit, living in northeastern Prussia, on a very cold winter day, adopted the then prevailing temperature as the zero point of his scale. He divided his thermometer into 212 equal degrees between his zero and the boiling point of distilled water. The freezing point of water on the Fahrenheit scale is situated at 32° F. There are, therefore, the following rules for changing one of the three scales into another:

1. n degrees Réaumur = $n \times \frac{5}{4}$ degrees Celsius
2. n degrees Celsius = $n \times \frac{4}{5}$ degrees Réaumur
3. n degrees Réaumur = $n \times \frac{9}{4} + 32$ degrees Fahrenheit
4. n degrees Celsius = $n \times \frac{9}{5} + 32$ degrees Fahrenheit
5. n degrees Fahrenheit = $n \div 32 \times \frac{4}{9}$ degrees Réaumur
6. n degrees Fahrenheit = $n \div 32 \times \frac{5}{9}$ degrees Celsius

The following table gives the comparative values in the three systems of thermometers:

Cels.	Fahr.	Réau.	Cels.	Fahr.	Réau.	Cels.	Fahr.	Réau.
−40	−40	−32	22	71.6	17.6	84	183.2	67.2
−39	−38.2	−31.2	23	73.4	18.4	85	185.0	68.0
−38	−36.4	−30.4	24	75.2	19.2	86	186.8	68.8
−37	−34.6	−29.6	25	77.0	20.0	87	188.6	69.6
−36	−32.8	−28.8	26	78.8	20.8	88	190.4	70.4
−35	−31	−28	27	80.6	21.6	89	192.2	71.2
−34	−29.2	−27.2	28	82.4	22.4	90	194.0	72.0
−33	−27.4	−26.4	29	84.2	23.2	91	195.8	72.8
−32	−25.6	−25.6	30	86.0	24.0	92	197.6	73.6
−31	−23.8	−24.8	31	87.8	24.8	93	199.4	74.4
−30	−22	−24	32	89.6	25.6	94	201.2	75.2
−29	−20.2	−23.2	33	91.4	26.4	95	203.0	76.0
−28	−18.4	−22.4	34	93.2	27.2	96	204.8	76.8
−27	−16.6	−21.6	35	95.0	28.0	97	206.6	77.6
−26	−14.8	−20.8	36	96.8	28.8	98	208.4	78.4
−25	−13	−20	37	98.6	29.6	99	210.2	79.2
−24	−11.2	−19.2	38	100.4	30.4	100	212.0	80.0
−23	− 9.4	−18.4	39	102.2	31.2	101	213.8	80.8
−22	− 7.6	−17.6	40	104.0	32.0	102	215.6	81.6
−21	− 5.8	−16.8	41	105.8	32.8	103	217.4	82.4
−20	− 4	−16	42	107.6	33.6	104	219.2	83.2
−19	− 2.2	−15.2	43	109.4	34.4	105	221.0	84.0
−18	− 0.4	−14.4	44	111.2	35.2	106	222.8	84.8
−17	1.4	−13.6	45	113.0	36.0	107	224.6	85.6
−16	3.2	−12.8	46	114.8	36.8	108	226.4	86.4
−15	5.0	−12.0	47	116.6	37.6	109	228.2	87.2
−14	6.8	−11.2	48	118.4	38.4	110	230.0	88.0
−13	8.6	−10.4	49	120.2	39.2	111	231.8	88.8
−12	10.4	− 9.6	50	122.0	40.0	112	233.6	89.6
−11	12.2	− 8.8	51	123.8	40.8	113	235.4	90.4
−10	14.0	− 8.0	52	125.6	41.6	114	237.2	91.2
− 9	15.8	− 7.2	53	127.4	42.4	115	239.0	92.0
− 8	17.6	− 6.4	54	129.2	43.2	116	240.8	92.8
− 7	19.4	− 5.6	55	131.0	44.0	117	242.6	93.6
− 6	21.2	− 4.8	56	132.8	44.8	118	244.4	94.4
− 5	23.0	− 4.0	57	134.6	45.6	119	246.2	95.2
− 4	24.8	− 3.2	58	136.4	46.4	120	248.0	96.0
− 3	26.6	− 2.4	59	138.2	47.2	121	249.8	96.8
− 2	28.4	− 1.6	60	140.0	48.0	122	251.6	97.6
− 1	30.2	− 0.8	61	141.8	48.8	123	253.4	98.4
0	32.0	0	62	143.6	49.6	124	255.2	99.2
1	33.8	0.8	63	145.4	50.4	125	257.0	100.0
2	35.6	1.6	64	147.2	51.2	126	258.8	100.8
3	37.4	2.4	65	149.0	52.0	127	260.6	101.6
4	39.2	3.2	66	150.8	52.8	128	262.4	102.4
5	41.0	4.0	67	152.6	53.6	129	264.2	103.2
6	42.8	4.8	68	154.4	54.4	130	266.0	104.0
7	44.6	5.6	69	156.2	55.2	131	267.8	104.8
8	46.4	6.4	70	158.0	56.0	132	269.6	105.6
9	48.2	7.2	71	159.8	56.8	133	271.4	106.4
10	50.0	8.0	72	161.6	57.6	134	273.2	107.2
11	51.8	8.8	73	163.4	58.4	135	275.0	108.0
12	53.6	9.6	74	165.2	59.2	136	276.8	108.8
13	55.4	10.4	75	167.0	60.0	137	278.6	109.6
14	57.2	11.2	76	168.8	60.8	138	280.4	110.4
15	59.0	12.0	77	170.6	61.6	139	282.2	111.2
16	60.8	12.8	78	172.4	62.4	140	284.0	112.0
17	62.6	13.6	79	174.2	63.2	141	285.8	112.8
18	64.4	14.4	80	176.0	64.0	142	287.6	113.6
19	66.2	15.2	81	177.8	64.8	143	289.4	114.4
20	68.0	16.0	82	179.6	65.6			
21	69.8	16.8	83	181.4	66.4			

INDEX TO AUTHORS

M

N

O

P

R

S

GENERAL INDEX

C

D

E

F

G

M

Q

R

S