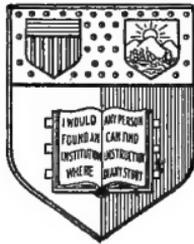


LABORATORY DIRECTIONS
FOR
BEGINNERS
IN BACTERIOLOGY
BY
MOORE



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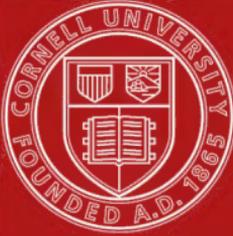
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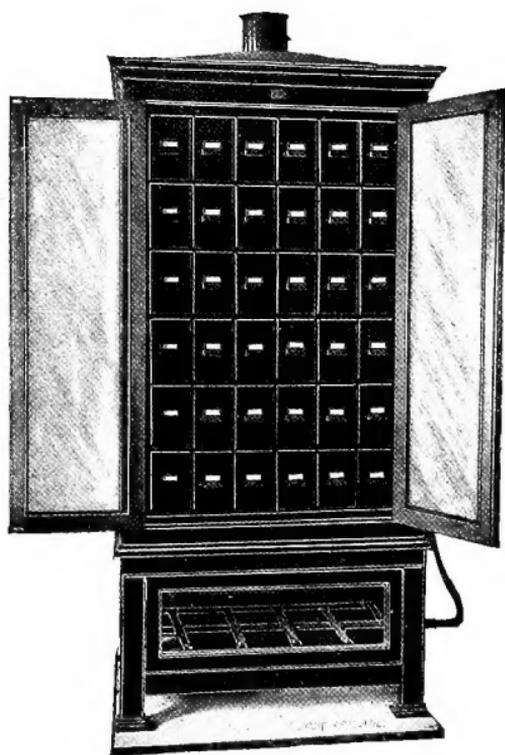
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AN APARTMENT INCUBATOR

LABORATORY DIRECTIONS

FOR BEGINNERS IN

BACTERIOLOGY

*An Introduction to Practical Bacteriology for Students
and Practitioners of Comparative and of
Human Medicine*

BY

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SECOND EDITION, ENLARGED AND REVISED

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PREFACE TO THE SECOND EDITION

THE call for a second edition of these Laboratory Directions has come in such a short time that many of the difficulties encountered in the preparation of the first edition still remain. The choice of subject-matter and the selection of methods for a short elementary laboratory course become more and more difficult with the rapidly increasing developments in bacteriology. The recognized etiological importance of a number of bacteria which formerly were considered of little significance necessitates, for the best results, an extension of a knowledge of bacteriology beyond the differential characters and properties of a few pronounced pathogenic species.

Experience with the first edition has clearly demonstrated the advantage to both student and teacher of specific directions for a working basis in carrying out the various procedures in a laboratory course. The exercises have been considerably modified, four new ones added, and a few references appended for the purpose of aiding students in familiarizing themselves with the current literature on the subject.

In revising these exercises new text and reference books have been freely consulted. Valuable suggestions have also been received from a number of teachers and investigators. I am especially indebted for such assistance to Dr. Theobald Smith of Harvard University, Dr. Erwin F. Smith of the United

States Department of Agriculture, and to Mr. Raymond C. Reed and Mr. Floyd R. Wright, Instructors in the Department of Bacteriology in Cornell University. Suggestions and criticisms which may tend to increase the usefulness of these outlines are cordially invited.

V. A. M.

ITHACA, N.Y.,

June, 1900.

PREFACE TO THE FIRST EDITION

It has been found desirable to provide the student, just beginning the study of bacteriology, with a somewhat detailed outline of the work to be done at each laboratory session. The selecting of the particular things to be done and the choosing of methods to be followed are difficult tasks. The assigning of directions for doing work under assumed conditions must necessarily partake of the empirical, and often fail. It is evident, however, that practical bacteriology must, if successfully taught, be cast in a somewhat definite form in order that the student may come to a knowledge of the fundamental principles underlying the subject in its twofold capacity, that of a pure science and of a useful art.

These outlines are intended either to serve simply as a guide through an introductory laboratory course preparatory to independent research work, or to form the basis for the application of the principles of bacteriology in the practice of human or of comparative medicine. They aim to impart a technical and working knowledge of certain of the more essential methods and to develop a definite knowledge of a few important species of bacteria. During the past year, they were furnished the students in mimeographed sheets, but after making the changes suggested by this application it seems desirable to put them in a more convenient form. In adjust-

ing the amount of work for each exercise to the necessary limitations of time and facilities, I am indebted to Mr. Raymond C. Reed, Instructor in this Department, for much valuable assistance. I wish also to thank Professor Charles Wright Dodge of the University of Rochester for helpful suggestions. Should these outlines fall in the hands of other teachers or workers in this subject, criticisms are cordially invited.

V. A. M.

ITHACA, N.Y.,
August, 1898.

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A LIST OF THE MORE IMPORTANT TEXT AND REFERENCE BOOKS

- Principles of Bacteriology. By A. C. Abbott.
- A Manual of Bacteriology. By Surgeon-General George M. Sternberg.
- A Text-book of Bacteriology. By Surgeon-General George M. Sternberg.
- Text-book upon the Pathogenic Bacteria. By Joseph McFarland.
- Laboratory Work in Bacteriology. By F. G. Novy.
- A Manual of Bacteriology. By Herbert U. Williams.
- Bacteriology in Medicine and Surgery. By William Hallock Park.
- Applied Bacteriology. By Pearmain and Moor.
- A Manual of Bacteriology, Clinical and Applied. By Richard T. Hewlett.
- A Text-book of Bacteriology and Infective Diseases. By Edward M. Crookshank.
- Manual of Bacteriology. By Muir and Ritchie.
- Micro-organisms and Disease. By E. Klein.
- Bacteria and their Products. By Sims Woodhead.
- Manual of Bacteriological Technique and Special Bacteriology. By Thomas Bowhill.
- The Principles of Bacteriology. By F. Hueppe.
- Elements of Clinical Bacteriology. By Levy and Klemperer.
- Micro-organisms in Water. By P. and G. C. Frankland.
- Bacteriological Diagnosis. By J. Eisenberg.
- Die Mikroorganismen. By C. Flügge.
- Grundriss der Bakterienkunde. By C. Fraenkel.
- Lehrbuch der bakteriologischen Untersuchung und Diagnostik. By Heim.
- Bakteriologische Diagnostik. By Lehmann and Neumann.
- System der Bakterien. By W. Migula.
- Mikrophotographischer Atlas der Bakterienkunde. By Fraenkel und Pfeiffer.
- Taschenbuch für den bakteriologischen Praktikanten u. s. w. By R. Abel.
- Jahresb. u. d. Fortsch. d. path. Mikroorganismen. By Baumgarten.
- Précis de Microbie. By Thoinot et Masselin.
- Précis de Bactériologie clinique. By Wurtz.
- Microbiologie Vétérinaire. By Mosselman and Liénaux.
- Traité de Bactériologie. By E. Macé.

JOURNALS AND PERIODICALS OF SPECIAL VALUE TO THE STUDENT OF BACTERIOLOGY

Centralblatt für Bakteriologie, Parasitenkunde u. Infektionskrankheiten.

Journal of Pathology and Bacteriology.

Zeitschrift für Hygiene u. Infektionskrankheiten.

Annales de l'Institut Pasteur.

Archives de Med. Experimentale et d'anat. Pathologique.

The Journal of Experimental Medicine.

The Johns Hopkins Hospital Bulletin.

The Journal of the Boston Society of Medical Sciences.

Annual Reports of the American Public Health Association.

All Standard Medical and Veterinary Journals.

Important articles on various topics in bacteriology frequently appear in journals on sanitary engineering, botany, chemistry, general biology, reports of city and state Boards of Health, United States Government Reports (especially those of the Bureau of Animal Industry and of the Marine Hospital Service), State Experiment Station Bulletins, and reports of scientific societies.

BOOKS VALUABLE FOR METHODS AND FORMULÆ

von Kahldeu. Pathological Histology.

Lee. Microtomet's Vade-Mecum.

Lafar. Technical Mycology.

Saccardo. Chromotaxia seu Nomenclator Colorum. Patavi, 1894.

APPARATUS AND MATERIAL

A. Apparatus in Laboratory for General Use. This includes the apparatus and chemicals to be used in common by all students. It consists of pans and brushes for cleaning test tubes and other glassware, meat mincer and press, large and small water baths, steam sterilizers, hot-air sterilizers, incubators, thermometers, thermostats, gas burners, balances, leveling tripods, Wolffhügels' or other apparatus for aids in counting colonies, micrometers, metric rules, burettes, tripods, funnels, beakers, pipettes, graduates, glass tubing, and rods. The chemicals necessary for carrying on the work, such as various acids and alkalies, disinfectants, alcohol, aniline dyes; those articles needed in the preparation of culture media, such as salt, peptone, agar, gelatin, meat extract, sugars, litmus and other indicators, and filter paper. Fresh meat, eggs, milk, and potatoes will be furnished as needed. It also includes color charts and the more important books of reference.

B. Apparatus furnished for Individual Use. The various appliances used by each student and for which he becomes personally responsible. It comprises a microscope with sub-stage condenser, two oculars (1 and 2 in.) and three objectives ($\frac{2}{8}$, $\frac{1}{8}$, and $\frac{1}{1\frac{1}{2}}$ inch), a bottle of immersion oil, a tripod magnifier, 75 small test tubes, 30 large test tubes, 10 fermentation tubes, 18 Petri dishes, 3 Erlenmeyer flasks, 5 one-ounce bottles

for reagents and stains, 6 pipettes with rubber bulbs to fit bottles, 1 platinum-wire loop, 1 platinum-wire needle, 3 tin cups for holding cultures, 3 tin boxes for holding test tubes, 1 block for holding reagent bottles, 1 glass slide with ring attached for hanging-drop preparations, 1 tin tray for cover-glass preparations, 2 solid watch-glasses, 2 Stender dishes for used slides and cover-glasses, and a glass box for clean cover-glasses. Each working table is provided with a reserve-flame gas burner (Bunsen), glass jars for waste, and stands for holding culture tubes. Requisite amounts of absorbent cotton, lens paper, and towels are furnished when needed.

C. Material to be provided by Each Student. A box of slides and cover-glasses ($\frac{3}{4}$ inch square cover-glasses preferred; they must be between .12 and .18 mm. in thickness), a slide box for permanent preparations, gummed labels, preferably with name printed upon them, for slides and cultures, a Faber's blue pencil for marking on glass, fine forceps for handling cover-glasses, and paper for laboratory notes with manila cardboard covers.

LABORATORY MAXIMS

1. See that the working table, instruments, and all pieces of apparatus used are thoroughly cleaned at the close of each exercise.

2. Unless otherwise directed, all cultures, other than those in gelatin, are to be grown in the incubator.

3. Gelatin cultures should not be put into the incubator except for special purposes not described in these directions.

4. In opening tubes of media or cultures, always flame the open end of the tube immediately after withdrawing the plug. If the tubes have been standing for some time, the surface of the plug should be flamed before drawing it out. Never allow the tube end of the plug to touch, while out of the tube, any article by which it could become contaminated. It should be held by the top between the fingers.

5. In making transfers, the tubes should be held as nearly in the horizontal position as possible. Cultures should not be opened in currents of air.

6. In every case where a platinum wire loop or needle is used for making cultures or withdrawing media it should be carefully heated in a gas flame both immediately before and after using. The heated wire must be allowed to cool before making cultures.

7. In making plate cultures, work as much as possible under a hood and in still air.

8. If by accident, a drop or more of a culture should be spilled upon the table or floor, pour over it a sufficient quantity of a disinfectant (corrosive sublimate solution 1-1000, or a 5% solution of carbolic acid) to completely cover the infected area. After this has acted for ten minutes wipe it up and boil or burn the cotton or cloth. If any of the culture should drop on the hands or clothing, a disinfectant should be applied immediately.

9. In sterilizing culture media, always see that there is enough water in the pan of the steam sterilizer or in the water bath before lighting the gas. Do not put the media in a sterilizer and leave the laboratory.

10. Always disinfect, by boiling, all cultures before cleaning the tubes or plates containing them. (A liberal supply of cleaning mixture can be used to advantage in some instances for destroying cultures.)

11. At the beginning of each laboratory session read the directions for the next exercise in order to be able to make any preliminary preparations which may be required.

12. Careful notes should be taken on all observations made in the study of cultures and preparations made from them.

LABORATORY BACTERIOLOGY

EXERCISE I

CLEANING GLASSWARE

1. It is necessary that the glassware employed should be thoroughly cleaned before it is used. Several special methods have been suggested for this purpose, but the one frequently employed by chemists seems to be the most easily handled and quite as efficient for general use as the more elaborate, specialized processes. It consists in applying the chromic acid cleaning mixture after washing the tubes and flasks with water. It is sometimes necessary to employ other methods for cover-glasses which are to be used in staining bacteria where a mordant is required. Only one of these special methods will be given here.

2. **Work for this Exercise.** — Clean all of the glassware, test tubes, fermentation tubes, flasks, Petri dishes, and reagent bottles assigned.

Put the slides and cover glasses in the cleaning mixture; they can be rinsed and wiped later.

3. **Methods to be followed in cleaning the different apparatus:** —

(a) *Test tubes.* Wash the tubes carefully with soap and water, using the test tube brush. After washing, stand them in a glass jar (aquarium) and fill them to within about 2 cm. of the top with cleaning mixture¹ (this will be kept in stock in the labora-

¹ *Formula for chromic acid cleaning mixture.* Dissolve 80 grams of potassium dichromate ($K_2Cr_2O_7$) in 300 c.c. of warm water; when all of the $K_2Cr_2O_7$ is dissolved and the solution cooled, add it slowly, with con-

tory). After it has acted for from 10 to 20 minutes, pour it out of the tubes into the bottle originally containing it. Rinse the tubes thoroughly in running tap water until all color disappears, and then in hot water, and drain them, using individual drainage board from locker. After they are dry, wipe the outside of the tubes with a slightly dampened cloth.

(b) *Fermentation tubes.* Treat these in the same manner as the test tubes, excepting in the use of the brush, which must be omitted.

(c) *Flasks.* Wash the flasks thoroughly with soap and water. Then fill them with the cleaning mixture and allow it to act for at least 10 minutes, after which it is to be poured back. Rinse the flasks thoroughly in the same manner as the test tubes and drain them. When dry, the outside should be wiped with a damp cloth.

(d) *Petri dishes and reagent bottles.* Thoroughly wash the Petri dishes and reagent bottles in hot soap-suds, after which rinse them separately in hot water. Wipe the Petri dishes dry with a soft cloth and drain the bottles. The cleaning mixture need not be used.

(e) *Cover-glasses and slides.* Drop the cover-glasses singly into a glass jar containing cleaning mixture and allow them to remain there for 24 hours or longer. Pour off the cleaning mixture and rinse the cover-glasses in boiled water until all of the color disappears, then cover them with alcohol until needed, when they can be wiped with a soft linen cloth or with lens paper. After they are wiped out of the alcohol, place them in a Petri dish without the cover, and heat them in the dry air sterilizer at a temperature of 160–180° C. for one hour.

After they have cooled, replace the cover and allow the cover-glasses to remain in the Petri dish (a glass jar or other closed dish may be used) until used. (When a drop of water or bouil-

stant stirring, to 460 c.c. of concentrated sulphuric acid. Store the mixture in a glass-stoppered bottle. The liquid will be quite thick with small crystals. When the crystals are used up, the liquid should be discarded.

lon is spread upon a properly cleansed cover-glass it will not roll up in droplets, but remain in a thin, even layer on the surface.)

Treat the slides with the cleaning mixture in the same manner as the cover-glasses. They may, however, be wiped directly out of the rinsing water. Slides can often be cleaned very satisfactorily by washing them in hot soap-suds, rinsing them in water, and wiping them with a soft cloth.

(f) *Cleaning used culture apparatus.* Place the tubes, flasks, or Petri dishes containing old cultures in a water bath, cover them with water, add a little sal soda (about an ounce to the gallon of water), and boil for 20 minutes. Pour off the water and empty the tubes, after which again boil them for 5 minutes in clean soap and water. Then wash and treat with the cleaning mixture the same as the new tubes. Cultures of spore-bearing pathogenic bacteria, such as those of anthrax, should be destroyed by heating in the autoclave at a temperature of at least 110° C. for half an hour before the tubes are emptied and washed.

4. A Method for Cleaning Cover-glasses for Flagella Stain. — For this work the ordinary method of cleaning cover-glasses is not sufficient, although the heating will often give a perfectly satisfactory cover-glass. The following treatment was highly recommended to me by Dr. Erwin F. Smith. First clean the cover-glasses by the ordinary method, after which boil them in an agate cup or glass beaker in a 10% solution of caustic soda for 5 minutes. After cooling, rinse the cover-glasses thoroughly in distilled water, place them in a beaker and cover them with a 1% solution of hydrochloric acid, heat to the boiling point, and allow to stand for several hours (over night or longer). Then pour the acid off and rinse the cover-glasses several times in distilled water, and finally in alcohol. Wipe them out of the alcohol as they are needed.

5. Laboratory Notes. — In this and all subsequent exercises careful notes should be taken on the work done. When, how-

ever, as in this exercise it consists simply of carrying out directions, a simple statement that the work as directed was completely or partially done, as the case may be, is all that is necessary.

In all other cases describe fully the work performed and observations made.

The notes should be as brief as completeness will permit. They should be legibly written and the technical terms peculiar to the subject in hand should be correctly used. The notes are to be handed to the instructor each week for examination and correction. When returned, all corrections should be carefully noted and similar errors should be avoided thereafter.

EXERCISE II

PLUGGING THE TUBES AND FLASKS AND STERILIZING
THE GLASSWARE

6. After the tubes and flasks are cleaned they must be plugged. The plugged tubes and flasks and the Petri dishes, all of which are to be used for holding culture media or in making cultures, must be sterilized before they can be used. The plugs should be neatly made and of the proper length and firmness. The best quality of absorbent cotton is ordinarily used for this purpose, although common cotton is employed in some laboratories. If the latter is used, it should be first heated to a very slight browning in the hot-air sterilizer. This drives off the oil and kills the spores which it might contain. Glassware is sterilized with dry steam or by means of dry heat, *i.e.* in the hot-air sterilizer. (See method for sterilizing apparatus and instruments in text-books.)

7. **Work for this Exercise.** — Plug all of the tubes and flasks with absorbent cotton and sterilize them, together with the Petri dishes, in the hot-air sterilizer. After they are sterilized, store them in the locker until they are needed. The Petri dishes must not be opened until they are used.

8. **Plugging the Tubes and Flasks.** — For this purpose absorbent cotton is used. The rolls of absorbent cotton are cut in short segments of from 5 to 7 cm. in length. A piece of this narrow strip of sufficient length to give cotton enough for the plug is torn off. The quantity varies, of course, with the size of the mouth of the tube or flask, but a little experience will enable one to estimate the quantity quite accurately. The edges of the piece of cotton torn off are turned in and it is rolled up to form a firm plug which should snugly fit the neck of the tube or flask. It should be inserted into the tube for about 2 cm. and the end should be nearly flat and smooth.

The projecting part should be about the same length and be of equal firmness. (For method of closing the tubes more securely, see paragraph 22.)

9. Sterilizing Glassware.—Place all the tubes, flasks, and Petri dishes in the hot-air sterilizer, close the door tightly, and light the gas. Heat the air in the sterilizer to a temperature of from 135 to 150° C. and keep it there for one hour. The temperature should not be allowed to go above 150° C. Turn the gas off, and when the temperature of the air in the sterilizer goes down to or below 45° C. the door may be opened and the apparatus removed.

EXERCISE III

THE PREPARATION OF BOUILLON

10. Bouillon is the liquid medium most commonly employed in cultivating bacteria. It is practically a beef tea containing peptone. There are several methods recommended for making it. (1) It may be made directly from simple meat infusion or (2) it may be made from meat extract. The meat infusion is prepared either by allowing finely chopped lean meat mixed with twice its quantity of distilled or filtered and boiled tap water (2 c.c. of water for each gram of meat) to stand in a cool place for from 12 to 18 hours, or the mixture of meat and water may be heated with frequent stirring at a temperature of 65° C. for a short time (one hour). Each has its advantages. When meat extract is used in place of the meat infusion, the bouillon does not seem to be a favorable culture fluid for certain bacteria. In making bouillon therefore it becomes necessary to determine the kind (whether from meat infusion or extract) and the method of preparing it to suit the conditions in hand. It is sometimes desirable in bacteriologic investigations to resort to all of these methods. For the routine work in the laboratory, bouillon prepared directly from the meat by macerating it for a short time at a high temperature (65° C.) is very satisfactory. The addition of peptone and the neutralization of the liquid is the same in both cases.

Bouillon is used as the nutritive base in preparing agar and gelatin. On this account the large quantities are stored in flasks. (For other methods see text-books. Also Jour. of the Am. Public Health Asso., Jan. 1898, p. 77.)

REFERENCES. — Chapters on making culture media in text-books, p. xi., Jour. of the Am. Public Health Asso.,¹ Oct., 1895. *Ibid.*,

¹ In June, 1895, a convention of bacteriologists was called in New York City under the auspices of a committee of the American Public Health

Jan., 1898, p. 77. Smith, *The Jour. of Exper. Med.*, Vol. III. (1898), p. 647.

11. Work for this Exercise.— Make 1000 c.c. of bouillon and distribute it as follows:—

Put $\frac{5}{7}$ c.c. in each of 10 small sterile test tubes.

Put 300 c.c. in each of 2 large (500 c.c.) Erlenmeyer flasks, and the remainder in a third flask.

Put $\frac{5}{7}$ c.c. of distilled water in each of 5 small sterile test tubes and sterilize them with the bouillon. (They are to be used subsequently in place of bouillon in making dilutions.) Label the tubes, "Sterile distilled water." All media required to carry out the directions will be furnished by the instructor excepting such as the student is directed to make in Exercises III., IV., and X.

12. The Preparation of Bouillon.— Take 500 grams of lean beef, remove all fat, and grind it in a sausage machine or have it minced at the butcher shop. Place the minced meat in an agate iron dish and add 1000 c.c. (2 parts water to one of meat) of clear boiled water, cooled to 65° C., and thoroughly stir with a glass rod. Then macerate it with frequent stirring in a water bath at a temperature of 60° C. for 1 hour after the temperature of the meat and water reaches that of the water outside. Remove the meat by straining the liquid through a piece of cheese cloth. For this a stout iron meat press is desirable. The liquid should equal in quantity the amount of water used; if it does not, add distilled or clear boiled water to make it

Association. One of the questions which was carefully discussed at that meeting was that of culture media. The papers and discussions were referred to a committee of nine bacteriologists with instruction to report at a subsequent time. At the meeting of the American Public Health Association in 1897, this committee made its report (printed in January number of its *Journal* for 1898). It covers very carefully the methods for preparing various culture media which are recommended for standard preparations, thereby tending to insure uniformity in results. This report is a most important publication on bacteriologic technique, "with special reference to greater uniformity in the description and differentiation of species."

up to that amount. To this meat infusion add 1% peptone (Witte's) and $\frac{1}{2}$ % sodium chloride. Add enough of a normal solution of sodium hydrate to give the liquid a faintly alkaline reaction. In this work the alkalinity can be determined by the use of sensitive litmus paper. (For neutralizing culture media for special or research work, see Appendix I., p. 126.) The infusion is then boiled in a water bath for three-quarters of an hour, and allowed to cool. When cool, filter it through ordinary filter paper. The filtrate should be perfectly clear. The color will vary according to the amount of blood pigment in the meat used, and according to the length of time it is steamed or boiled, *i.e.* on the amount of material precipitated out. After filtering, distribute the bouillon in tubes and flasks (see above). Stand the tubes containing the bouillon in a wire basket for sterilization. Sterilize them by boiling in a closed water bath or steaming in the Arnold's steam sterilizer for 30 minutes,¹ the time to be computed from the time the water boils or the temperature in the steamer reaches 99°. The flasks of bouillon should be boiled or steamed for 20 minutes on each of the two succeeding days (certain anaërobic bacteria may not be destroyed by this treatment). When they have cooled, the outside of the tubes should be carefully wiped with a moist

¹ The customary method of sterilizing culture media is to steam or boil it for about 10 minutes on each of 3 consecutive days. This was found very troublesome by the students, and feeling that it was not necessary, a long series of test experiments was made by Mr. R. C. Reed, who found that 1 boiling or steaming for 30 minutes gave just as good results as the customary 3 boilings. As the media is not used for 2 or 3 days after its sterilization, during which time it is kept in an incubator, the method is well suited to student laboratories, not for the reason that it saves time in preparing the media, but it relieves the congestion in the sterilizer and appreciably aids the student. *When sterilized by this method the media must not be inoculated for several days after its preparation.*

Media can be quickly sterilized by means of the autoclave when the temperature is raised from 110 to 115° C. While this method is quick and convenient, the high temperature seems to be detrimental to media for certain pathogenic bacteria. The autoclave, however, is quite extensively used.

cloth and placed in the incubator until the next laboratory day. Then carefully examine them, and if any of the tubes are contaminated, that is, if the liquid is clouded or has a membrane on the surface, they must be rejected. Label all others, and place them in the locker.

13. Labelling Media and Culture Tubes.—Stick on each tube of media, about 3 cm. from the top, an adhesive, white label about 2 cm. square. On the upper line should be written the name of the medium, and the date of its preparation. Thus, Bouillon, 13-VII-1900. When the tube is used, the name of the organism or material with which it is inoculated, together with the date of inoculation, should be written on the lower lines. This applies to all media and tube or flask cultures.

EXERCISE IV

THE PREPARATION OF AGAR AND GELATIN

14. Of the solid media employed in cultivating bacteria, agar and gelatin are most commonly used. They depend for their nutritive properties largely upon the bouillon from which they are made, the agar and gelatin forming simply the solidifying elements. The striking difference between the two is that the gelatin melts at the body temperature, whereas the agar is not quickly liquefied below the boiling point. For this reason gelatin is not used as a solid medium for cultivating bacteria at a high (body) temperature. There are several processes for preparing these media, but the addition of the dry agar and gelatin to bouillon (12) either immediately after it is filtered or later after it has been sterilized and stored in flasks seems to be the most convenient procedure. The agar itself is usually neutral in reaction, but the gelatin often has a decidedly acid reaction. This necessitates the careful testing of the reaction of the two media although the bouillon is neutral or slightly alkaline.

REFERENCES. — Chapters on culture media in text-books on Bacteriology, p. xi., The preparation of nutritive agar. Am. Mic. Jour., May, 1890. A rapid method of making agar-agar, Johns Hopkins Hospital Bulletin, No. 24, July-Aug., 1892. Jour. of the Am. Public Health Asso., Jan., 1898.

15. **Work for this Exercise.** — Prepare 300 c.c. of agar and 300 c.c. of gelatin, i.e. start with 300 c.c. of bouillon for each. There will be considerable shrinkage owing to the amount lost on the dishes, filter, etc., so that the quantities of media will be appreciably less than this amount. Distribute each medium as follows: —

Put 7 c.c. in each of 10 small sterile test tubes.

Put 12 c.c. in each of 12 large sterile test tubes.

Put the remainder in a small sterile flask.

16. The Preparation of Nutrient Gelatin. — Take a flask of bouillon containing 300 c.c. and pour it into a small agate iron dish and add 30 grams of sheet gelatin which has been cut into small pieces, heat the bouillon, with frequent stirring, in a water bath until the gelatin is dissolved. Allow it to cool to a temperature between 45° and 50° C. and then add the white of one egg and mix it thoroughly by stirring, or better by pouring the gelatin many times from one flask or beaker to another. After the egg albumen is completely diffused, return the liquid gelatin to the large covered water bath and boil until the egg albumen is coagulated. This takes about 20 minutes. It is now ready for filtering, which must be done while the gelatin is hot. Filter through properly folded but ordinary filter paper, first moistened with boiling water. (For illustrations and directions for folding filter paper, see Abbott's "Prin. of Bact.," p. 97. Filter paper already folded may be procured.) Distribute the filtrate as directed. In pouring the gelatin into the tube use a small beaker or graduate and see that the gelatin does not touch the sides of the upper part of the tube. Stand the tubes in a wire basket and sterilize them by boiling in a closed water bath or by steaming in the Arnold's steam sterilizer for 30 minutes. The small flasks can be sterilized in the same manner. Place tubes and small flasks in the incubator and allow them to remain there for two days. If the gelatin in any of the tubes becomes cloudy, the medium in those tubes must be rejected. Carefully wipe all of the other tubes with a moist cloth, label, and place them in the locker where they can be kept until used.

17. The Preparation of Nutrient Agar. — Weigh out 3 grams of agar and cut it into small pieces with a pair of scissors. Put the finely cut agar into an agate iron dish and add 50 c.c. of distilled water and boil it over a gas flame with constant stirring, to prevent scorching, until the agar is dissolved, giving a thick homogeneous pasty substance. Pour 300 c.c. of bouillon (11) from a flask into the cup containing the dissolved agar.

Place the dish containing the mixed agar and bouillon in a closed water bath and boil for 20 minutes. Then cool it to a temperature between 45 and 50° C. and add the white of one egg and thoroughly mix it in the liquid agar. This is easily accomplished by pouring it a number of times from one beaker to another. When the egg albumen is dissolved, the agar is returned to the water bath and boiled vigorously until the white of the egg is coagulated. This usually takes about 20 minutes. Filter the agar immediately, while hot, through ordinary filter paper which has been moistened with boiling water. Distribute the filtrate in small and large tubes, as directed. Sterilize, label, and store the agar in the same manner as the gelatin.

EXERCISE V

INOCULATING TUBES OF BOUILLON, AGAR, AND GELATIN

18. Work for this Exercise. — Inoculate one tube of bouillon, two (one inclined, the other not) of agar, and one of gelatin from a culture of *Bacillus coli communis*, which will be furnished.

Wipe the slides. Transfer the cover-glasses from the cleaning mixture to water and alcohol and wipe them. Place them in a Petri dish and heat them as directed in paragraph 3 *e*.

Read the chapters in one or more of the text-books on inoculating media or making tube cultures.

19. Inoculating Bouillon. — In making this culture, carefully remove the plug from the tube of bouillon by first twisting it around to detach any adhesions and then pull it straight out. Pass the open end of the tilted tube quickly through the gas flame. The plug, which has meantime been carefully held, is partially replaced and the tube returned to its stand. Treat the tube containing the culture (which has been furnished) in the same manner. Then place the two tubes side by side between the thumb and forefinger of the left hand, palm facing upward, and grasp them about the middle of the upper half (see Fig. 38, p. 108, Crookshank). Sterilize the platinum loop by passing it through the gas flame, care being taken that the handle is also flamed for a distance of at least 15 cm. Then carefully remove the plugs from the tubes and hold them between the fingers in such a manner that the tube ends, projecting outward, will not touch anything during the inoculation process. Insert the wire loop carefully into the culture and transfer a loopful of the culture to the tube of bouillon and gently rinse it from the loop. The loop is then withdrawn, the plugs replaced in their respective tubes, and the loop flamed and put aside. Label the freshly inoculated tube with the name of the organism, source, and date. Stand it in a tray or

cup and place it in the incubator.¹ This should be kept at a temperature between 35 and 37° C. The organism thus transferred should multiply so that on the following day the liquid will be cloudy. It is then a bouillon culture of *B. coli communis*.

20. Inoculating Tubes of Agar. — *Ordinarily the agar is inclined before it is inoculated.* In this case it is spoken of as inclined or slant agar. Occasionally the agar is inoculated without inclining it. Cultures made in this manner are spoken of as “stab” or “stick” cultures. (1) Inclined or slant agar. Stand a tube of agar in a wire basket in a water bath and boil it until the agar is liquefied. (To save repeating this it is well to incline the agar in several tubes which can be kept for future use, but after the slants have been made for a long time it is better to boil and reslant them, especially if they are to be used for organisms which do not grow well on a dry surface.) Lay the tubes on a tray, the top resting on the side of the tray so that the surface of the agar will be about 4 cm. long, and allow it to cool. In placing the tubes the label should be up. When the agar has set, it is ready for use. It is inoculated precisely as the bouillon, excepting the loopful of culture is drawn over the inclined surface instead of being thrust into the medium as in the bouillon. Label and place it in the incubator with the inoculated bouillon tube. On the following day there should be a grayish white growth on the surface of the agar covered by the loop. This is an agar culture of *B. coli communis*. (2) Stick cultures. These are made with a platinum needle in the uninclined agar. The impregnated needle is pushed down through the centre of the agar. In all other respects this culture is made like the slant agar culture.

¹ For illustrations and descriptions of different kinds of incubators, see text-books. It is desirable to note especially the various burners and thermo-regulators employed to heat and to regulate the temperature of the incubators. Considerable information can also be acquired by carefully looking through the catalogues of manufacturers and dealers in bacteriologic apparatus. Copies of some of these will be found on the reference book shelves.

21. Inoculating Tubes of Gelatin. — Tube cultures in gelatin are usually made without inclining the gelatin, *i.e.* stick cultures. The tube of gelatin is inoculated in the same manner as the stick culture in agar. This tube is to be placed in the locker, as the gelatin will melt at the incubator temperature. The growth will appear in about two days along the needle track. This is a gelatin culture of *B. coli communis*.

22. Sealing Culture Tubes. — It is often desirable to seal cultures to prevent their drying out quickly. A convenient method, and one which has long been in use in some laboratories, is to boil a small quantity of paraffin in a small agate iron dish, and while it is still hot carefully dip the tube end of the plug into it and quickly replace it in the tube. The paraffin on cooling adheres to the sides of the tube, forming a tight plug. When the tube is to be opened, the end must be warmed slightly before the plug can be withdrawn. The plugs should be paraffined and the sterility of the tubes determined before they are used for cultures.

EXERCISE VI

THE EXAMINATION OF CULTURES

23. In studying cultures of bacteria, it is necessary to observe very carefully (1) the macroscopic appearance of the growth in or upon the media, (2) the microscopic appearance of the bacteria in (*a*) the living condition (hanging-drop preparation), and (*b*) in the dead and stained condition (cover-glass preparation), and (3) the effect of the growth of the bacteria upon the chemical and the physical properties of the medium. To determine these, the cultures must be kept under observation for several days and often for several weeks. A careful record should be made of the changes observed in the appearance of the cultures. Illustrate with drawings.

24. Work for this Exercise.—Examine carefully and describe fully the appearance of the bouillon, agar, and gelatin cultures made in Exercise V.

Determine the reaction of the bouillon culture and note whether there is any change in its consistence (viscosity).

Make a hanging-drop preparation from each culture and examine and describe the appearance of the bacteria in each.

Make a drawing of the gelatin and slant agar cultures and also of a few of the bacteria in one of the hanging-drop preparations.

Read the paragraphs in one or more text-books on the examination of cultures and hanging-drop preparations.

25. Suggestions for the Macroscopic Examination of Cultures.—The external appearance of cultures should be observed and noted on the day after they are made and on each succeeding day until the growth ceases. In bouillon cultures note the appearance of the liquid, whether uniformly, faintly, or heavily clouded, turbid, clear, or clouded with flocculent

masses held in suspension, the quantity and nature of sediment, and the presence or absence of a membrane. The reaction of the liquid should be taken and its consistence noted. The odor should be determined. In agar cultures the extent of the growth (feeble, moderate, or vigorous), its color, form, and surface appearance (dull or glistening), should be observed. The character of the growth in the condensation water should also be noted. In stab cultures the appearance of the growth both on the surface and along the needle track should be described. In gelatin, the absence or the presence and extent of liquefaction should be noted in addition to the features already referred to for the stab agar cultures.

26. Testing the Reaction of Liquid Cultures. — Place a small piece of each of the red and blue litmus paper in a solid watch glass. With the platinum loop carefully place a drop of the culture on each piece of the paper. After recording the reaction produced, neutral, acid, or alkaline with the degree, cover the paper with a disinfectant (a solution of corrosive sublimate 1 to 1000). After it has acted for about 10 minutes, empty it with the paper into the waste jar and wash the watch glass.

27. To determine the Viscidity. — (1) Bouillon cultures. Insert the platinum loop into the liquid and carefully withdraw it. The approximate degree of viscosity can be determined by the extent of the adhesion of the liquid to the loop, and the length of the thread-like filament drawn out. By gently shaking the tube a viscid sediment will rise up, appearing as a somewhat twisted, tenacious cone with its apex reaching to or near the surface. A friable sediment will break up and become disseminated through the liquid upon agitation. (2) Agar and gelatin cultures. Touch the surface growth with the end of the platinum needle, and if it is viscid, a thread-like string will be drawn out. Note whether the growth is pasty or friable.

28. Making Hanging-drop Preparations.— (1) From a bouillon culture. Place a clean cover-glass on the tray. With the loop, remove a drop of the liquid culture and place it on the middle of the cover-glass. With a pair of fine forceps invert the cover-glass over the glass ring fixed to a slide for this purpose. The surface of the ring should previously be moistened with liquid vaseline to prevent the cover-glass from sliding. The preparation is then ready for examination. Examine it first with the high power dry lens and then with the oil immersion objective. (For directions in the use of the microscope, see "The Microscope" by Professor S. H. Gage.) (2) From cultures on solid media. On account of the very large number of bacteria in the growth on solid media it is necessary to separate them in a clear liquid. Take a cover-glass as before and place a loopful of sterilized water or bouillon on the centre. With the platinum needle touch the surface growth very gently with the end of the needle and carefully rinse it in the drop of liquid on the cover-glass. From this point the examination is the same as with the liquid culture. Upon examination, if the bacteria are so numerous that the individual organisms cannot be clearly distinguished, *i.e.* separated from each other, the preparation must be rejected and another one made, using a smaller quantity of the growth. After examination, the cover-glasses should be placed at once in a glass jar containing a strong disinfectant (5% carbolic acid, 1 to 1000 corrosive sublimate solution, or a strong solution of a mineral acid).

29. Suggestions for the Microscopic Examination of Living Bacteria.—In examining the bacteria, as they appear under the microscope in the hanging-drop preparation, the following features should be observed: Are the individual bacteria spherical, rod-shaped; or spiral in form? Are they single or united in pairs, masses or clumps, or in shorter or longer chains? For this determination it is better to examine the organisms near the edge of the drop. Are they motile, that

is, do the individual bacteria move from one point in the field to another? To determine this the centre of the drop is better. Clearly distinguish between genuine motility and a simple dancing motion (the Brownian movement). Determine the presence or absence of spores. These are bright, highly refractive bodies either within or outside of the bodies of the bacteria. If present, they can usually be seen in both positions. Is there any evidence of a capsule around the bacteria?

EXERCISE VII

MAKING AND STAINING COVER-GLASS PREPARATIONS,
AND FORMULÆ FOR STAINING SOLUTIONS

30. Work for this Exercise. — Make 2 cover-glass preparations from each of the cultures made in Exercise V. and stain one of each with alkaline methylene-blue and the other with carbol fuchsin. Describe the appearance of the bacteria and make a drawing of a few individual bacteria from the preparations made from the agar culture.

Preserve a cover-glass preparation mounted in balsam and labelled to accompany notes.

Prepare the staining fluids used in this exercise from the formulæ given.

Read the paragraphs in the text-books on making and staining cover-glass preparations.

31. Making Cover-glass Preparations. — (1) From bouillon cultures. Place 2 clean cover-glasses on the tray. With the loop remove a drop of the bouillon culture and spread it in a thin layer over about two-thirds of the surface of the cover-glasses. One loopful will ordinarily make from 2 to 4 preparations. Allow the liquid to dry on the cover-glasses in the air. When dry, fix the bacteria to the cover-glasses by passing them, film upward, three times through the middle of the upper half of the gas flame. Each passage (complete circle) should not occupy more than 1 second. After fixing, they are ready for staining. (2) From cultures on solid media (agar, gelatin, potato, serum, etc.). Place the cover-glasses on the tray, and on the centre of each put a drop of sterile water or bouillon. With the needle touch the surface growth of the culture and then gently rinse the end of the needle in the liquid on the covers. Spread the liquid on the covers as before.

From this point the procedure is the same as that for the preparations made from the bouillon culture.

32. Staining Bacteria in Cover-glass Preparations. — (1) With alkaline methylene-blue. With the pipette place a few drops of the staining solution on the film side of the fixed preparation, which is either held horizontally with the fine forceps or left resting on the tray. Allow the stain to act for 2 or 3 minutes. Then carefully rinse off the stain in water, holding the cover firmly by one edge with the forceps. After thoroughly rinsing, place the preparation, film downward, on a clean slide and dry the upper surface with a piece of filter paper. It is now ready for the microscopic examination. Use first the dry lens ($\frac{1}{8}$ in. obj.) and then the oil immersion objective. If the specimen is a good one and it is desirable to preserve it, wipe off the drop of oil with a piece of lens paper, run a drop of distilled water under the cover-glass which will float it, when it can be easily removed with the forceps. Place it on the tray, film upward, and when dry mount it in alkaline Canada balsam.

(2) With carbol fuchsin. Cover the film on the cover-glass with the stain and allow it to act for about 1 minute. Then rinse it thoroughly in water, after which cover it with $\frac{1}{10}\%$ solution of acetic acid or strong (95%) alcohol. Allow this to act from 5 to 10 seconds, and again thoroughly rinse in water and examine as above. (For other decolorizers, see text-books.)

Upon examination the preparation should be free from deposits or stained background. The bacteria should, as a rule, be isolated and distinct; unless they are the preparations are not satisfactory.

Cover-glass preparations of bacteria are permanently mounted in the same manner as similar preparations made from the blood or other tissues in histology, the process being to put a drop of balsam on the centre of the slide and place the preparation, film downward, over it and apply slight pressure. Label the preparation, giving the name of the organism, its

source (kind of culture, tissue, etc., from which the preparation was made), stain used, and date. If the specimen is not preserved, the slide and cover-glass should be cleaned for future use.

33. Suggestions concerning the Microscopic Examination of Stained Preparations of Bacteria. — In the examination of the bacteria in the stained condition the following points, and perhaps others, should be observed and noted. (1) Concerning their morphology. Are they spherical, rod-shaped, or spiral? Are they separated or united in clumps or chains? If rod-shaped, are the ends pointed, round, or square? Are the bacteria all of the same form and size? Note the presence or absence of spores and capsules. (2) Concerning their reaction to staining fluids. Do they stain uniformly or irregularly? Do they stain deeply or faintly? Is the centre lighter than the periphery? Is there an unstained central band and deeply stained ends (polar stain)? Do all of the bacteria take the stain alike?

34. Staining Solutions. — The basic aniline dyes are used in staining bacteria. There is a large number of these, and there are several formulæ for preparing staining solutions from each. Further, as will be seen from the chapters on staining bacteria in the text-books, there are several methods of applying these stains. In an introductory course, however, it is impossible to try them all, and consequently those are described which seem to be the best adapted for general use.

In addition to the ordinary staining solutions and methods there are special processes for certain species, such, for example, as the tubercle bacterium, and still others for staining certain parts of many bacteria, such as the flagella on motile forms, the spores in spore-bearing organisms, and the capsule on certain other species. There is a large number of these special methods, but in this course only one of each will be given. These will be taken up in connection with the study of the bacteria requiring them.

35. Formulæ for Staining Solutions. — The dyes here used are methylene-blue, gentian-violet, methyl-violet, and basic fuchsin. For the other dyes see text-books.

ALKALINE METHYLENE-BLUE (LOEFFLER'S)

Saturated alcoholic solution of methylene-blue	30 c.c.	6
Caustic potash 1 % solution	1 c.c.	6
Distilled water	100 c.c.	26

The saturated alcoholic solution of the methylene-blue (or of any of the dyes) is prepared by pouring the dye into a clean bottle and filling it about one-fourth full. Then fill the bottle with strong (95 % or absolute) alcohol, cork tightly, shake, and allow it to stand for 24 hours. If at the end of that time the dye is entirely dissolved, add more, shake thoroughly, and allow it to stand for another day. Repeat this procedure until there is a permanent sediment of undissolved coloring matter in the bottom of the bottle. Then label. (The saturated solution will be kept in stock in the laboratory.)

CARBOL FUCHSIN (ZIEHL'S SOLUTION)

Fuchsin (dry)	1 gram.
Alcohol (absolute)	10 c.c.
Carbolic acid, 5 % solution	100 c.c.

Dissolve the fuchsin in the alcohol, after which add the carbolic acid solution. Instead of using the dry fuchsin and alcohol, 11 c.c. of a saturated alcoholic solution of fuchsin may be used.

It is more convenient for each student to prepare the following: —

Saturated alcoholic solution of fuchsin	2.5 c.c.
Carbolic acid, 5 % solution	20 c.c.

ANILINE METHYL-VIOLET (EHRlich-WEIGERT)

Saturated alcoholic solution of methyl-violet	11 c.c.
Absolute alcohol	10 c.c.
Aniline water	100 c.c.

36. Aqueous Solutions. — Aqueous solutions of methyl-violet, gentian-violet, fuchsin, and the other aniline dyes are prepared by adding 1 c.c. of the saturated alcoholic solution of the desired dye to 20 c.c. of distilled water. This will impart a decided color to the liquid so that a pipette full will be barely transparent.

The true aqueous solutions are made by dissolving the dyes in water, but these are weak and not so effective as those prepared from the alcoholic solutions. These solutions deteriorate in a short time. The carbol fuchsin and alkaline methylene-blue will keep a little longer, but they require to be filtered occasionally.

37. Making Aniline Water. — Aniline water is a saturated aqueous solution of aniline oil. It is prepared by adding 1 c.c. of aniline oil to 20 c.c. of distilled water and shaking frequently for from 15 to 30 minutes. It is convenient to use a stoppered vial or large test tube for mixing it. Filter through a moistened filter paper. The filtrate should be perfectly clear. If it is cloudy, it should be refiltered before using. This is used in preparing the aniline water dyes, such as methyl-violet, gentian-violet, etc.

38. Gram's Method of Staining Bacteria. — Prepare the cover-glass preparations as already described. Stain them in gentian-violet aniline water or in a saturated alcoholic solution of gentian-violet in 5% carbolic acid in the proportion of 1 to 20 for from 5 to 7 minutes. Rinse in water and transfer them to a watch glass containing Gram's solution until the color becomes quite black. This requires from 1 to 2 minutes; then place the preparations in a watch glass containing alcohol and allow them to remain there until the color has almost entirely disappeared, or has become a pale gray. Rinse in water and examine at once, or allow them to dry and mount in balsam. (Sections of tissues must be dehydrated and cleared before mounting.)

Formula for Gram's solution (Lugol's) : —

Iodine	1 gram.
Potassium iodide	2 grams.
Distilled water	300 c.c.

Certain bacteria stain deeply and retain the coloring matter when treated by this method, while others are decolorized by the alcohol. On this account some investigators consider it an important aid in the differentiation of certain bacteria.

EXERCISE VIII

MAKING PLATE AND ESMARCH ROLL CULTURES

39. The general principle underlying the separation of bacteria by means of plate and roll cultures is to dilute the substance containing the bacteria so that the individual organisms will be separated from each other by an appreciable distance and then fixed in a solid medium where each organism can multiply into a growth or colony without coming in contact with any other organism or colony. For this purpose agar and gelatin are used. Originally Koch employed a rectangular piece of glass for holding the layer of medium, and protected it from contamination by putting it under a bell jar. Later Esmarch introduced the "roll culture" method, which was extensively followed, until the Petri dishes were introduced. Since that time they have been largely used in place of the Koch plate and Esmarch tube. On this account the plate cultures of to-day are usually made in Petri dishes. The roll culture is also used.

Plate cultures are employed for two distinct purposes, viz. (1) to isolate bacteria in order to obtain pure cultures from the isolated colonies, and (2) to determine how many bacteria there are present in a given quantity of a liquid such as water, milk, or blood. In this exercise the object is to separate the bacteria to obtain isolated colonies. For quantitative work, see Exercises LX. and LXI.

40. Work for this Exercise.—Make a series of 3 agar plates, one of 3 gelatin plates, and a series of 3 gelatin roll cultures (Esmarch rolls) from the bouillon culture of *B. coli communis* (18). Place the agar plates in the incubator and the gelatin plates and rolls in a locker for that purpose.

Reëxamine all the cultures made in previous exercises and add to the laboratory notes a description of any changes in

their appearance. The notes should contain a detailed record of the cultures made in this exercise.

Read carefully the paragraphs in the text-books on making plate and roll cultures.

41. Making Agar Plate Cultures. — Take three large tubes of agar, stand them in a water bath, and boil until the agar is liquefied. Then cool by standing the tubes with a thermometer in a cup of water at a temperature of about 50° C. As the temperature rises, add a little cold water. When the temperature of the agar reaches that of the water, and the temperature of the whole has lowered to 40° C., the agar is ready for use. For convenience in labelling, number the tubes 1, 2, and 3.

Place 3 sterilized Petri dishes on the levelling tripod and adjust it by means of a spirit level. With the wire loop proceed by the same method as followed in making bouillon cultures. Take 1 loopful of the bouillon culture and place it in agar tube No. 1 and mix by carefully shaking it. Flame the wire and transfer 2 loopfuls of agar from tube 1 to tube 2 and mix as before. Again flame the loop and transfer 3 loopfuls from tube 2 to tube 3 and mix as with tubes 1 and 2. After the tubes are inoculated, pour the agar into the Petri dishes. In doing this remove the plug, carefully flame the mouth of the tube, and after quickly cooling raise with the left hand the edge of the cover on one side of the Petri dish sufficiently to allow of inserting the mouth of the tube. After the agar is poured out of the tube replace the cover immediately. Label, and number the Petri dishes to correspond with the dilutions in the tubes, thus, plate 1 is from tube 1, plate 2 is from tube 2, and plate 3 is from tube 3. In making the dilutions it is important that the wire loop should be flamed after making each transfer.

42. Making Gelatin Plate Cultures. — These are prepared precisely as the agar plates with these exceptions. (1) The gelatin is liquefied at a temperature of 45° C. (2) The plates when made are to be kept in the locker the same as the gelatin

stab cultures. (3) In hot weather it is sometimes necessary to put a piece of ice in the reservoir under the glass plate on the levelling tripod to congeal the gelatin.

The directions given above for making the dilutions are applicable only when the original culture is moderately clouded. If there are comparatively very few bacteria in the liquid, a larger quantity of the culture will be necessary. If there are many more, as in turbid bouillon or slant agar culture cultures, it will be necessary to take a much smaller quantity for the first dilution. It is often desirable to make the first dilution in a tube of sterile water or bouillon instead of gelatin or agar, and to make 2 rather than 3 plates. It is sometimes desirable to make 4 or more cultures.

43. Making Esmarch Roll Cultures. — For this purpose gelatin is ordinarily used. Agar does not adhere readily to the sides of the tubes. It is sometimes used. Take the desired number of large tubes of gelatin, liquefy, inoculate, label, and number the dilutions as in making gelatin plate cultures. Place a block of ice about 6 inches long in an agate iron or glass tray. Melt a slight nearly horizontal groove in the ice with a test tube containing hot media or water. The inoculated tubes are tipped and rolled so that the liquid gelatin moistens the inside of the tube to within about a centimetre of the plug. Then roll the tube rapidly in the groove on the ice until the medium becomes solid. The gelatin should not come in contact with the plug. In rolling the tube the plugged end should always project beyond the ice. See illustration in text-books. (Fig. 29, p. 133, Abbott.)

EXERCISE IX

THE EXAMINATION OF PLATE CULTURES AND THE
MAKING OF SUBCULTURES FROM COLONIES

44. In practical bacteriologic work, plate cultures are made use of in determining (1) the number of bacteria there is in a given substance, (2) the different species of bacteria present, and (3) the character of the growth in a colony of the organism in question. Other important facts, such, for example, as the relative number of each species of bacteria, or the difference in the appearance of the surface and deep colonies, are learned through this process. The plate culture, therefore, is one of the most important single methods employed in isolating and studying bacteria.

45. Work for this Exercise. — Examine carefully and describe the plate cultures made in Exercise VIII. If the agar plates do not have colonies, or if the colonies are so numerous that they cannot be counted on any of the plates, make the cultures over again, and give an explanation in the notes of this exercise for the failure to obtain good results.

Make a hanging-drop preparation from a colony from an agar plate, and one from a colony from a gelatin plate, and examine them microscopically. Describe the appearance of the bacteria in each.

Make a cover-glass preparation from each of the same colonies and stain each with carbol fuchsin. Examine each preparation carefully, and make a drawing of a few of the isolated bacteria. Describe (33) the appearance of the bacteria in these preparations.

Inoculate a tube of bouillon, one of agar, and one of gelatin from a well-isolated colony on one of the agar plates.

46. Suggestions for the Examination of the Plate and Roll Cultures. — Observe the general appearance of the plates, note

whether the colonies are well isolated or run together (confluent); describe the appearance of the individual colonies, (a) those on the surface, (b) those in the depth of the medium. Indicate their shape (round, lenticular, flat, convex, or spherical). Are the edges sharply defined? Is the margin even or irregular? Give their size (diameter in millimetres), and indicate their color (determine shade from a color chart¹) and consistence. Do the surface colonies adhere to the medium or can they be easily removed? Examine them with a low power lens and describe the surface markings if any. Also indicate the difference in color as observed with the unaided eye and with the microscope.

47. Estimating the Number of Colonies on Plates.— If the number of colonies is not large (not to exceed 100), they may all be counted and the exact number recorded. This may be done with the third plate. When the number is larger, it is more convenient to divide the total area into smaller ones and count the number of colonies in each of several (20 to 40) of the small areas; add these together and divide the sum by the number of areas counted. The quotient gives the average number on one area; multiply this quotient by the number of areas containing colonies, and the product will be the number of colonies on the plate. This latter process, however, gives the approximate number only.

For dividing the area of the plate into smaller, equal areas, it is convenient to use Wolffhügel's counting apparatus. This was devised more particularly for square or oblong plates (Koch). In counting the colonies on the Petri dishes Parkes'² scheme modified by Jeffers³ is more suitable. It consists of a disk about 20 cm. in diameter divided into areas of a square centimetre each. Place the Petri dish over the disk, taking care that it is accurately centred.

¹ Saccardo, *Chromotaxia seu nomenclator colorum*.

² Parkes, *Journal of Pathology and Bacteriology*, Vol. IV., p. 173.

³ Jeffers, *Journal of Applied Microscopy*, Vol. I., No. 3, 1898.

Count the number of colonies in several (20 to 40) of the areas and multiply the mean number by the number of areas covered. This product gives the approximate number of colonies on the plate.

48. Making Subcultures from Colonies.—Select the tubes of media to be used, and flame the mouths as heretofore described. Select a colony as well isolated from all others as possible. With the left hand carefully raise the edge of one side of the cover of the Petri dish, and, while holding it, touch the colony with the needle, replace the cover, take up the tube of medium, and inoculate it. If bouillon is used first, a tube of agar or gelatin can be inoculated immediately afterward without recharging the needle. If more cultures are to be made, it is necessary to again charge the needle from the colony. If the plate is to be rejected, the cover can be entirely removed in the beginning. The newly inoculated tubes or subcultures should be labelled and treated according to the directions heretofore given for handling cultures. These inoculated tubes should be pure cultures. It sometimes happens, however, that what appears to be a single colony consists of the growths of two organisms. If these should be of different species, the cultures made from the colony would probably be impure. These impure growths (apparently single colonies) frequently develop on plate cultures exposed to the air for some time. The single particles of dust often carry two or more bacteria.

EXERCISE X

THE PREPARATION OF CERTAIN SPECIAL MEDIA

49. In studying the properties of bacteria it is desirable to cultivate them on a number of different media. Bouillon, agar, and gelatin are most commonly used, but others are necessary in determining the cultural peculiarities and important biochemic properties of the organism in question. The cultivation of bacteria upon these media may be regarded in the light of a test, to determine the presence or absence of certain properties or powers possessed by the bacterium in question. Thus, for example, whether the species in hand will coagulate the casein in milk, produce gas in media containing saccharose, grow on potato, etc. The number of these tests which have been used and recognized as important is quite large, but in a short course only those possessed of special differential value can be tried. In describing a new species, or identifying any of the carefully described ones, it is important to know at least some of these cultural peculiarities and biochemic properties. For this reason it is necessary to learn the method of preparation and the use of certain of these media. The more important of such media are included in this exercise.

In addition to the above, a few species of bacteria require particular kinds of media for their diagnostic or most differential growth. Among these are the specific organisms of glanders, diphtheria, and tuberculosis. The preparation of these particular media will be considered in connection with the study of the organisms requiring them.

50. **Work for this Exercise.**—Prepare for culture media 5 tubes of potato, 5 tubes of milk, 5 tubes of litmus milk, 5 tubes of glucose agar, 5 tubes of glycerin agar, 3 fermentation, and 5 small test tubes of bouillon containing glucose, the same number and kinds of tubes containing lactose, and the

same containing saccharose. (The agar and the sugar-free bouillon necessary in the work of this exercise will be furnished by the instructor.)

Read carefully the paragraphs in the text-books on the preparation and use of these media.

51. Preparation of Potato for a Culture Medium.—Select 3 medium sized potatoes, thoroughly wash and rinse in boiled water, and cut out, with a cutter made for this purpose, cylinders 3 to 4 cm. long (oblong rectangular pieces cut with a knife will do quite as well). Ordinarily 2 cylinders can be cut from each potato. These can be cut obliquely, giving 2 pieces each. All of the skin must be removed. Wash the potato cylinders in cold, running water for 5 minutes (a longer time is preferable) and place them in test tubes of the proper size (large or small according to size of cutter used), and add about 1 c.c. of water to each tube. Sterilize them by discontinuous boiling or steaming for 20 minutes each day for 3 consecutive days. Wipe, label, and store in locker.

52. Preparation of Milk for a Culture Medium.—Place about ~~100~~ c.c. of fresh milk in a beaker in the ice box and allow it to stand for from 10 to 15 hours. Then carefully remove the cream. It is well to filter the milk through a thin layer of absorbent cotton to remove any masses of cream. The reaction should be tested, and if strongly acid it should be rejected or made 1.5% acid to phenolphthalein by the addition of $n/10$ sodium hydrate. Distribute the skimmed milk in small test tubes (7 c.c. in each) and sterilize by discontinuous steaming in the same manner and for the same length of time as the potatoes. Wipe, label, and store the tubes in locker.

53. Preparation of Litmus Milk for a Culture Medium.—This is prepared the same as the milk medium with the addition of enough of an aqueous solution of litmus to impart a decidedly blue color to the milk. Sterilize, wipe, label, and store the same as the milk. The litmus solution will be furnished.

54. Preparation of Glucose (Grape Sugar) Agar.—Prepare

100 c.c. of agar (17). Take ³⁰ ~~50~~ c.c. of it for glycerin agar, and to the remainder add 1% glucose. Dissolve the powdered glucose in about 5 c.c. of boiled, hot water before adding it to the liquid agar. After thoroughly mixing distribute it in small, sterile test tubes. Sterilize, wipe, label, and store the same as ordinary agar.

55. Preparation of Glycerin Agar. — Take ¹⁵ ~~50~~ c.c. of the agar prepared above, and add ³⁰ 5% of pure (c. p.) glycerin. Thoroughly mix it with the agar, after which distribute it in tubes. Sterilize, label, and store as ordinary agar.

56. Preparation of Glucose Bouillon. — This is used in the fermentation tube. Take ~~100~~ c.c. of sugar-free peptonized bouillon (59), and add 1 gram of pure grape sugar (glucose). After it is dissolved and thoroughly disseminated through the bouillon by stirring or pouring, distribute the bouillon in 3 fermentation tubes, filling completely the closed branch and the open bulb about half full, and put 7 c.c. in each of 5 small sterile test tubes. Sterilize by discontinuous steaming for 20 minutes each day for 3 consecutive days. The tubes should be wiped, labelled, and placed in the locker until needed for use.

57. Preparation of Lactose Bouillon. — This is prepared by adding ^{8 gm.} 1% of pure lactose (milk sugar) to the peptonized sugar-free bouillon. It is necessary that the bouillon, used does not contain muscle sugar. After adding the lactose, which has been dissolved in a few c.c. of the bouillon and thoroughly mixing it in the bouillon, distribute in fermentation tubes and small test tubes, sterilize, label, and store the same as the glucose bouillon.

58. Saccharose Bouillon. — This is peptonized sugar-free bouillon to which 1% pure saccharose (cane sugar) has been added. It is prepared from bouillon free from muscle sugar in the same manner as lactose bouillon.

59. The Preparation of Sugar-free Bouillon. — Bouillon prepared by the ordinary method usually contains small quantities

of muscle sugar. To eliminate this, the following method has been recommended.¹ Beef infusion is inoculated in the evening with a rich fluid culture of some acid producing organism (*B. coli communis*) and placed in the incubator. The next morning the white of an egg is added, and the infusion is boiled and filtered. Peptone and salt are added as usual. It is boiled, filtered again, and distributed in tubes or flasks as desired, and sterilized the same as bouillon (12).

¹ Smith, *Journal of Experimental Medicine*, Vol. II. (1897), p. 543.

EXERCISE XI

INOCULATING SPECIAL MEDIA AND EXAMINING CULTURES

60. Work for this Exercise. — Inoculate a tube of potato, one of milk, one of litmus milk, one of glucose agar, a fermentation and test tube of glucose, one each of lactose and of saccharose bouillon. Label each, and place all of them in the incubator.

Examine microscopically the agar and bouillon cultures made from the colony on the agar (41). Examine and carefully describe the bacteria from each culture in (1) a hanging-drop preparation, and (2) stained cover-glass preparations. Stain a preparation with alkaline methylene-blue, one with carbol fuchsin, and one with an aqueous solution of methyl-violet. Make a careful comparison of the 3 preparations and note any difference in the appearance of the bacteria or in the degree of intensity of the stain. Preserve as a permanent specimen, to accompany the notes, a preparation stained with each of the dyes.

Prepare the aqueous solution of methyl-violet (36).

61. The Inoculation of Glucose Agar to determine the Power of the Organism to produce Gas. — Boil the tube of glucose agar in an open water bath until it is liquefied, then cool it down to a temperature of 40° C. and inoculate it with a loopful of the culture, carefully stir the agar with the loop, after which solidify it as quickly as possible. Label, and stand in the incubator.

62. The Use of Media containing the Sugars. — The sugars are employed as tests to determine whether the bacteria in question will ferment them producing acids. Some bacteria will produce gas as well as acids. The latter is determined in the sugar agar tubes.

In the fermentation tubes we can determine both of these properties and also the quantity of gas set free. It is easier,

however, to determine the acid and gas production in the test tubes than to use the fermentation tubes, and it is cheaper. It is convenient, therefore, to use these tubes with the sugar media as follows :—

1. If to determine power of organism to produce gas only use the agar tubes.

2. If to determine power of organism to ferment sugars producing acids only use test tubes of bouillon.

3. If to determine the quantity of gas produced and approximately its composition use the fermentation tube. In this exercise all three are called for.

EXERCISE XII

THE EXAMINATION OF CULTURES ON SPECIAL MEDIA

63. As certain of these media are used to determine the effect of the bacteria upon them, it is important to observe very carefully not only the appearance of the growth of the bacteria, but also their effect, if any, upon the medium on or in which they are growing. This is especially noticeable in the milk, litmus milk, and sugar bouillon cultures. The changes here are largely due to the action of the bacteria on the sugars or their power to produce alkali.

64. Work for this Exercise.— Examine and describe the cultures made on the special media in Exercise XI.

Examine the bacteria on the potato culture microscopically (1) in the fresh condition (hanging-drop preparation), and (2) in stained cover-glass preparations. Stain a preparation with ~~carbol fuchsin~~ and one with an aqueous solution of gentian-violet. Describe the appearance of the bacteria (33) and make a drawing of a few of them.

65. A Few Points to be observed in Studying Cultures on Special Media :—

(a) *Potato*. Note carefully the extent and color of the growth and its consistence.

(b) *Milk*. Note whether or not the general appearance and odor of the milk has been changed, observe whether the casein has been coagulated, giving a firm, solid coagulum, or precipitated. Is the coagulum covered with a liquid (serum)?, if so, is it clear or milky? Is there any appearance suggestive of saponification? Determine its consistence, chemical reaction as indicated by litmus paper (26), and give as descriptive a name as possible to its odor.

(c) *Litmus milk*. Note especially whether there has been

any change in color since inoculation. Observations similar to those on the plain milk should also be made.

(d) *Glucose agar*. Note the character and number of colonies within the agar, and the presence, if any, of gas bubbles. Are there few or many of them?

(e) *Bouillon containing sugars in test tubes*. Note carefully the appearance of the bouillon, but especially its chemical reactions as indicated by the litmus paper (26).

(f) *Bouillon containing sugars in fermentation tubes*. Observe the character of the growth in each tube (whether the liquid is faintly or heavily clouded, turbid, contains flakes, etc.), in (1) the open bulb and (2) the closed branch of the fermentation tube. Note the presence or absence of a membrane on the surface of the liquid in the open bulb. Is there a sediment in the bottom of the tube? If so, describe its general appearance and consistence. Note the presence or absence of gas in the closed branch. Indicate the quantity and note its rate of formation from time to time. Test the reaction of the liquid with litmus paper.

The fermentation tubes are also used to enable one to determine the quantity and kinds of gases produced and the aërobic or anaërobic tendencies of the organism. See Exercises XXIII, and XXIV.

EXERCISE XIII

THE EXAMINATION OF CULTURES (*Continued*)

66. Work for this Exercise. — Reëxamine the cultures made on special media and make notes on all changes which have occurred in their appearance.

✓ Examine microscopically in hanging-drop preparations the bacteria from the glucose-bouillon culture.

✓ Make 3 stained cover-glass preparations from the milk culture. Stain with the different dyes already used.

✓ Reëxamine all of the cultures previously made and make careful notes of any changes in appearance.

Reject all of the cultures made excepting those on agar from the colony, which should be preserved, and clean the tubes and Petri dishes (3, f).

67. Making Cover-glass Preparations from Milk Cultures. — Spread as thin a film as possible of the milk culture on the cover-glass and allow it to dry in the air. Immerse the preparation in a watch-glass, or other receptacle, containing a few cubic centimetres of ether and absolute alcohol in equal parts, which dissolves out the fat and fixes the film to the cover-glass at the same time. Then remove and, after the ether and alcohol have evaporated, stain as usual. The amount of albumen in the milk will usually cause a heavy background, which will require decolorizing with alcohol or weak acetic acid.

EXERCISE XIV

THE CLASSIFICATION OF BACTERIA

68. The term "bacteria" is a general and popular one used to designate a large group of microscopic plants, the *Schizomycetes*. These organisms, which are widely distributed in nature, have been classified into a certain few families and genera most of which have a large number of species. Many of these species have been described, but there are many which have not. In classifying the bacteria, the genera are based on morphologic characters; while, as a rule, the species are determined by means of their biochemic, physiologic, or pathogenic properties. Several systems of classification have been proposed, but the one which seems to be the most satisfactory is by Migula. This classification utilizes the morphology to such good advantage that its adoption seems desirable. It requires, however, some serious changes in the accustomed nomenclature. This, however, is true of any logical system. The restoration of the genus *Bacterium*, and the assigning to it of all non-motile, rod-shaped organisms, changes the genus of some of our most common pathogenic bacteria from *Bacillus* to *Bacterium*. The most conspicuous of these are the *Bacilli* of tuberculosis, glanders, and diphtheria, all of which are placed in Migula's classification in the genus *Bacterium*. The families and genera recognized by him are appended.

FAMILIES

- I. Cells globose in a free state, not elongating in any direction before division into 1, 2, or 3 planes . . . I. Coccaceæ. = *n* 
- II. Cells cylindrical, longer or shorter, and only dividing in 1 plane, and elongating to twice the normal length before the division.

- (1) Cells straight, rod-shaped, without sheath, non-motile, or motile by means of flagella 2. Bacteriaceæ. *family*
- (2) Cells crooked, without sheath 3. Spirillaceæ. *"*
- (3) Cells enclosed in a sheath 4. Chlamydo bacteriaceæ
- (4) Cells destitute of a sheath, united into threads, motile by means of an undulating membrane 5. Beggiatoaceæ.

GENERA

1. *Coccaceæ* *family name*

Cells without organs of motion.

- a. Division in 1 plane 1. Streptococcus. *Genera*
- b. Division in 2 planes 2. Micrococcus. *=*
- c. Division in 3 planes 3. Sarcina. *=* 

Cells with organs of motion.

- a. Division in 2 planes 4. Planococcus.
- b. Division in 3 planes 5. Planosarcina.

2. *Bacteriaceæ*

Cells without organs of motion 1. Bacterium. *=* 

Cells with organs of motion (flagella).

- a. Flagella distributed over the whole body 2. Bacillus. *=* 
- b. Flagella polar 3. Pseudomonas. *=* 

3. *Spirillaceæ* *family name*

Cells rigid, not snakelike or flexuous.

- a. Cells without organs of motion 1. Spirosoma.
- b. Cells with organs of motion (flagella).

- 1. Cells with 1, very rarely 2-3 polar flagella 2. Microspira.
- 2. Cells with polar flagella, in tufts of from 5-20 3. Spirillum. *S*

Cells flexuous 4. Spirochæta. *S*

4. *Chlamydo bacteriaceæ* *to*

Cell contents without granules of sulphur.

- a. Cell threads unbranched.

- I. Cell division always only in 1 plane . . . 1. Streptothrix.
- II. Cell division in 3 planes previous to the formation of conidia.
- (1) Cells surrounded by a very delicate, scarcely visible sheath (marine) . . . 2. Phragmidiothrix.
- (2) Sheath clearly visible (in fresh water) 3. Crenothrix.
- b.* Cell threads branched (pseudo branches) 4. Cladothrix.
- Cell contents containing sulphur granules 5. Thiothrix.

5. *Beggiatoaceæ*

Only 1 genus known (*Beggiatoa* Trev.), which is scarcely separable from *Oscillaria*. Character as given under the family.

Of these genera Streptococcus, Micrococcus, Bacterium, Bacillus, Microspira, and Spirillum contain the most important of the pathogenic bacteria. The familiar genus Staphylococcus of older classifications is included in the genus Micrococcus by Migula. It is important that the distinguishing characters of these genera be thoroughly learned.

REFERENCES. — Migula, Die natürlichen Pflanzenfamilien, Lieferung 129, Leipsic, 1896. Migula, System der Bakterien, 1897. Fischer, Jahrbücher für wissenschaftliche Botanik, Band XXVII., Erstes Heft.

69. Work for this Exercise. — Read the references on the morphology and classification of bacteria.

Learn from the text-books and lecture notes the more essential elements in the structure of bacteria.

Inoculate a tube of bouillon, one of agar, and one of gelatin (from cultures which will be furnished) with each of the following genera of bacteria.

- ✓ A streptococcus . . . *Streptococcus*
- ✓ A micrococcus *Micrococcus*

same
EXERCISE XV

THE MORPHOLOGY OF STREPTOCOCCUS AND

is by morphology MICROCOCCUS
is by special media.

70. Genera among bacteria are based on the gross morphology of the organisms. This is very largely true of all classifications. It is highly important, therefore, that the generic characters should be thoroughly learned. While the descriptive differences between a micrococcus and a bacterium seem to be clear there are many organisms where it is not so easy to decide in which genus to place them. The almost constant appearance of unexpected bacteria in septic infections and in diseased organs renders it exceedingly desirable that one should understand the fundamental elements of classification. We must remember that the problems of the practitioner are not all centred about known pathogenic forms like the organisms of tuberculosis and diphtheria; but they have to do with a great host of infecting bacteria, of which we know as yet but very little.

71. **Work for this Exercise.** — Carefully describe each of the ~~broth~~ cultures made in Exercise XIV.

Prepare and examine a hanging-drop preparation from each of the ^{*broth*} cultures, and describe the appearance (form) of the organisms in each. Indicate the morphologic characters by which each genus can be differentiated from the others.

Make a cover-glass preparation from ^{*broth*} each culture and stain with an aqueous solution of methyl-violet. Make a careful microscopic examination of each preparation and describe the bacteria in each.

Make careful notes on the appearance of the bacteria in each preparation and preserve a specimen of each to accompany notes.

Include in the notes the generic characters of each of these genera.

Measure carefully with the filar micrometer the length and thickness of ~~8~~⁴ individual bacteria in the stained preparation of the micrococcus. Record the measurements in microns (written μ). (For the use of the micrometer see appendix, also chapter on magnification and micrometry in "The Microscope," by Professor S. H. Gage.)

Inoculate a tube of bouillon, one of gelatin, and one of agar from a culture (furnished) of each of the following genera of bacteria.

- A micrococcus (staphylococcus form) . *Micrococcus* . . .
 A sarcina *Sarcina* . . .

EXERCISE XVI

THE MORPHOLOGY OF MICROCOCCUS (STAPHYLOCOCCUS FORM) AND SARCINA

72. Work for this Exercise. — Carefully examine and describe the culture made in Exercise XV.

Prepare and examine a hanging-drop preparation, and a stained (aqueous solution of methyl-violet) cover-glass preparation from each culture. Describe carefully the bacteria in each preparation, and measure with the filar micrometer a few individual organisms in each of the stained preparations.

Preserve a stained cover-glass preparation of each genus of bacteria to accompany notes. (If preferred, the student may use any of the other stains for the permanent mount.)

Include in the notes the generic characters of each of the genera studied in this exercise.

Inoculate a tube of bouillon and one of agar from a culture (furnished) of each of the following bacilli, viz. : —

Bacillus cloacæ (or other actively motile forms).

Bacillus lactis viscosus. causes rancidity in milk.

Bacillus subtilis.

EXERCISE XVII

THE MORPHOLOGY OF BACILLUS

73. The bacilli which are to be studied in this exercise exhibit in addition to the rod-shaped bodies the essential morphological variations of this genus, viz., number of flagella, capsules, and spores. The two latter are held in common with the bacterium. The staining of the organs of locomotion (flagella) and the spores will be taken up in separate exercises.

74. Work for this Exercise. — Carefully examine and study the cultures made in Exercise XVI., following the directions given for the examination of cultures and preparations in that exercise.

State fully in the notes the generic characters of the genus *Bacillus*.

Inoculate a tube of bouillon and one of agar from each of the cultures, which will be furnished, of a *Bacterium* and *Spirillum*. Place the inoculated tubes in the incubator.

EXERCISE XVIII

THE MORPHOLOGY OF BACTERIUM AND SPIRILLUM

75. Work for this Exercise. — Examine very carefully and describe fully the cultures of a Bacterium and a Spirillum made at the last exercise. *not motile, produce*

Make and examine, microscopically, hanging-drop and stained cover-glass preparations from each of the cultures. Describe the appearance of the individual bacteria in each.

Make a drawing magnified 1000 diameters of a few individuals from each of the stained preparations from the agar cultures.

State fully the characters of these two genera and mention the differential characters between Bacillus and Bacterium.

EXERCISE XIX

STAINING SPORES

76. In certain species of bacteria and under suitable conditions, there appear within the bacteria highly refractive bodies known as spores. The formation of spores is restricted to certain species. The spores are oval in form, and in old cultures they may often be found outside of the bodies of the organisms which produce them. They possess the power of resisting drying, heat, and unfavorable environment much longer than the bacilli themselves. They do not stain by the usual methods employed in staining bacteria so that special methods are required. Several processes have been proposed, but the one here given seems to be quite as efficient as any of the others.

Bacillus subtilis, or the hay bacillus, is one of the most widely distributed species of bacteria. It develops spores which can be readily detected either in fresh or stained preparations from cultures.

REFERENCES. — Methods for staining spores in text-books.

77. Work for this Exercise. — Examine and carefully describe the two cultures of *Bacillus subtilis* made in Exercise XVI.

Make a hanging-drop preparation from the bouillon and one from the agar culture and examine them microscopically. Describe the bacilli and observe carefully the appearance of the spores both within and outside of the organisms.

Make a cover-glass preparation from each culture and stain with alkaline methylene-blue. Examine carefully and note the appearance of spores which remain unstained. Make a drawing of a few of the bacteria containing spores.

Make a few (about 3) cover-glass preparations and stain them for spores.

Inoculate from a culture of *Bacillus cholerae suis* (furnished) a tube of agar and place it in the incubator for the next exercise.

78. A Method for Staining Spores. — Make a cover-glass preparation, dry, and flame as already described. Take the preparation by the edge with the fine forceps, cover the film surface with carbol fuchsin, and hold the preparation over the gas flame until steam is given off, then remove it for a few seconds and again heat it. Repeat the heating three or four times. After the stain has acted for from 3 to 5 minutes, rinse the preparation in water, and decolorize it by immersing it in a watch-glass containing about 3 c.c. of a 1% solution of sulphuric acid or of 95% alcohol. After about one-half minute remove the preparation and rinse it thoroughly in water. If it is not decolorized, repeat the bleaching process. This removes the coloring matter from the bodies of the bacteria, but leaves it in the spores. After thoroughly washing the preparation, counter stain it with a saturated aqueous solution of methylene-blue for about 30 seconds, rinse in water and examine. The spores should be stained red (with the fuchsin) and the rest of the organism should be colored blue.

There is a very satisfactory method recommended by Möller. For this and other methods for staining spores, see text-books on bacteriology.

EXERCISE XX

STAINING THE FLAGELLA ON MOTILE BACTERIA

79. The motile bacteria are provided with a variable number of long, hairlike appendages or flagella. These are invisible in the fresh preparation, and they do not stain by the ordinary methods. By special staining processes, however, their presence can be detected. Several methods have been proposed for staining these filaments, but nearly all of them are based on the use of a mordant. Curiously enough the value of each of these methods seems to rest largely on the skill of the individual using them, as some workers succeed with one method while others fail with it but obtain excellent results with one of the other processes. Although the flagella are known to be the organs of locomotion, they do not seem to be of any special morphological value in differentiating closely related species. They are, however, elements in the structure of motile bacteria, and their demonstration is much to be desired.

REFERENCES. — Chapters on staining flagella in standard textbooks. Moore, *A Review of the Methods, etc.*, *Am. Monthly Mic. Journal*, Vol. XII. (1891), p. 15. Moore, *The Character of Flagella, etc.* Wilder, *Quarter-century Book*, p. 339. Loeffler, *Centralblatt f. Bakteriologie, etc.*, Bd. VI. (1889), S. 209. Ferrier, *Archives de Méd. Exp. et d'Anat. pathologique*, T. VII. (1895), p. 58. Van Ermengem, reviewed in *Centralblatt für Bakteriologie, etc.*, Bd. XV. (1894), No. 24.

80. **Work for this Exercise.** — Make a cover-glass preparation from the growth on the agar culture of *Bacillus cholerae suis* made in Exercise XVII. and stain it with carbol fuchsin. Preserve this to compare with preparations stained for the purpose of demonstrating the flagella.

Clean about 20 cover-glasses after the special method for

flagella staining (4). Make about 10 cover-glass preparations on these from the agar culture and stain for flagella. Use Loeffler's method, but if it does not succeed, the second process may be tried.

81. Making Cover-glass Preparations for Flagella Stain.— Place 2 loopfuls of sterilized, distilled water or normal salt solution on the centre of the cover-glass. Gently touch the surface growth on the agar culture with the end of the platinum needle and immerse it in the water on the cover-glass without spreading the drop. The impregnated needle should carry bacteria enough for 3 or 4 preparations. Then place the tray of cover-glasses in the incubator to dry. The bacteria become disseminated throughout the water by means of their power of locomotion. When dry, they are ready for the staining treatment.

82. Staining the Flagella by Loeffler's Method.— The bacteria are fixed to the cover-glass by holding them, film upward, between the thumb and forefinger, over a gas flame for about a minute. They are then treated with the following mordant:—

Tannic acid, 20% solution	10 c.c.
Sulphate of iron, saturated solution	5 c.c.
Fuchsin, saturated alcoholic solution	1 c.c.

This should be filtered before using.

Place the fixed cover-glass preparation ~~in a large test tube,~~ cover it with the mordant, and carefully heat over a gas flame or in a water bath until steam is given off. Allow the mordant to act for from 3 ~~to 5 minutes.~~ Then remove the cover-glass with a bent wire loop and fine forceps and thoroughly rinse it in water. Then place it in a similar tube and cover it with carbol fuchsin for staining. Heat this as the mordant was heated and allow the stain to act for from 5 ~~to 10~~ minutes. Remove the cover-glass as before and thoroughly rinse in water. If the stain is too deep, decolorize by rinsing the preparation for a few seconds in alcohol and again in water. It is

then ready for the microscopic examination in water, or it may be allowed to dry and then be mounted in balsam. If the first preparation fails, add 2 drops of a 10% solution of sulphuric acid to the mordant.

The flagella should appear as fine, hairlike appendages radiating from the bacteria.

83. Staining the Flagella by Van Ermengem's Method. — The films are prepared as described above. Three solutions are necessary : —

Solution A (fixing bath).

Osmic acid, 2% solution	1 part.
Tannin, 10-25% solution	2 parts.

Place the films in this for 1 hour at room temperature, or heat in an oven for 5 to 15 minutes at 55° C. Wash the preparation with distilled water, then with absolute alcohol for 3 to 4 minutes, and again very thoroughly in distilled water. It is now ready to treat with Solution B.

Solution B (sensitizing bath). This is a 5% solution of silver nitrate in distilled water. Allow the films to be in this for from 2 to 3 minutes. Then without washing transfer to Solution C.

Solution C (reducing and strengthening bath).

Gallic acid	5 grams.
Tannin	3 grams.
Fused potassium acetate	10 grams.
Distilled water	350 c.c.

Keep in this for 1 to 1½ minutes. Wash, dry, and mount. It will also be found an advantage to use a fresh supply of Solution C for each preparation, a small quantity being sufficient. If overbrowned, the background will be too deeply stained ; if underbrowned, the flagella will be too faint.

EXERCISE XXI

STAINING TUBERCLE BACTERIA (BACILLI)

84. The stained tubercle bacterium possesses the power of retaining the coloring matter even when treated with a strong decolorizer, such as a solution of sulphuric or nitric acid. On this account staining has a high differential value which is made use of in identifying this organism. Thus, in the examination of sputum in cases of suspected tuberculosis, the object is to determine the presence of tubercle bacteria. As this organism is not easily cultivated, the staining process is very largely depended upon in making a differential diagnosis.

85. Work for this Exercise.— Make 4 cover-glass preparations from a culture of tubercle (furnished). Stain two of them with tubercle stain and carefully describe the appearance of the bacteria and illustrate them with a few drawings.

Stain two of the preparations after Gram's method.

Stain a cover-glass preparation of tubercular sputum (furnished).

Read the directions in the text-books for staining tubercle bacteria (bacilli).

86. Staining Tubercle Bacteria.— Prepare the cover-glass preparations from the culture of tubercle bacteria and flame them as already described. Stain in fresh carbol fuchsin. Place a few drops of the stain on the film side of the cover-glass and hold it over a flame with forceps until steam is given off. Allow the hot stain to act for from 3 to 5 minutes. Or the preparation may be floated on the carbol fuchsin in a watch-glass without heat. In this case it is allowed to act for from 10 to 15 minutes. The preparation is then rinsed in water and decolorized by treating it with a 10% solution of nitric or sulphuric acid for $\frac{1}{4}$ to 1 minute. It is again rinsed in water when it is ready for examination. It can be dried and mounted

permanently in balsam. The tubercle bacteria should be stained a deep reddish color. All other bacteria or animal tissue in the preparation should be unstained. If desired, a counter stain, such as alkaline methylene-blue, may be used after decolorizing. That is, the preparation should be again stained for about 1 minute in alkaline methylene-blue, rinsed in water, and examined as before. In these preparations the tubercle bacteria are red and the other organisms and cells are blue. A counter stain is of no value in preparations made from pure cultures or for simple diagnostic purposes. When a counter stain is desired Gabbett's decolorizing and counter-staining solution is very convenient.

Formula : —

Methylene-blue (powder)	2 grams.
10% sulphuric acid	100 c.c.

After staining with the carbol fuchsin treat the preparations with this mixture until the film has a faintly bluish tint. This solution decolorizes and counter stains at the same time. This organism, like some other pathogenic bacteria, takes the Gram stain. See Novy's "Laboratory Work in Bacteriology," p. 289, for a list of such organisms.

EXERCISE XXII

MAKING CULTURES OF ANAEROBIC BACTERIA

87. Certain bacteria will not grow in the presence of oxygen (atmosphere), and consequently they must be cultivated in a medium from which the air has been expelled, or in the presence of some neutral gas such as hydrogen. While certain bacteria, like those of symptomatic anthrax, tetanus, and malignant œdema, require the absence of oxygen, others, like *Bacillus subtilis*, will not multiply without it. There are, however, a large number of bacteria which are able to multiply independently of the presence or absence of this element. In reference to oxygen requirements bacteria are grouped as follows : —

Obligative aërobic bacteria — require oxygen.

Obligative anaërobic bacteria — require the absence of oxygen.

Facultative aërobic bacteria — grow best in the absence of oxygen, but will grow in the presence of air.

Facultative anaërobic bacteria — grow best in the presence of oxygen, but will grow in its absence.

There are several methods of cultivating anaërobic bacteria, but as a rule they are difficult and cannot be easily handled in an elementary course. Two of the simpler processes, however, will be tried.

88. Work for this Exercise. — Following the method of Liborius for cultivating anaërobic bacteria, inoculate a tube of agar from the culture furnished.

Inoculate 2 fermentation tubes from the same culture. One of the fermentation tubes should contain sugar-free bouillon (furnished), the other bouillon containing 1% glucose.

Inoculate for study at the next exercise 2 tubes of liquid agar (one plain and one containing glucose), a fermentation

tube of sugar-free bouillon, and one containing 1% glucose bouillon with *B. coli communis* from a culture furnished.

89. Liborius' Method. — Liquefy 2 tubes of agar and carefully pour them together. After this, boil the medium for at least 5 minutes to expel the air, cool it down to a temperature of 40° C. and then inoculate it from the culture of anaërobic bacillus (*B. —*) furnished, after which cool the medium rapidly by standing it in cold water until it is set. In inoculating the tube insert the loop nearly to the bottom and stir very gently. In making the inoculations care must be taken not to introduce air by shaking the liquid medium. Place the culture in the incubator.

90. The Fermentation Tubes for Anaërobic Bacteria. — If these tubes of bouillon have been properly sterilized, the closed branch is practically free from atmosphere. The obligatory anaërobe will grow in the closed branch only, while the facultative anaërobe will grow in both the open and closed parts. If the organism is a gas producer, the gas will force the cloudy liquid from the closed bulb into the open one, clouding the otherwise clear liquid. To avoid the possibility of error in interpreting these growths, it is well to inoculate a tube containing sugar-free bouillon, in which case the liquid in the open bulb should remain clear, as gas will not be formed.

These tubes are of equal value in testing obligatory and facultative anaërobic organisms.

EXERCISE XXIII

EXAMINATION OF CULTURES OF ANAËROBIC BACTERIA

91. Work for this Exercise. — Examine and carefully describe the appearance of the anaërobic cultures made in Exercise XX.

With the wire loop remove one of the colonies from the depth of the agar culture and examine it microscopically in (*a*) a hanging-drop preparation, and (*b*) a stained cover-glass preparation. Stain with carbol fuchsin. Examine microscopically in similar preparations the bacteria from one of the fermentation tubes. Describe the appearance of the bacteria in each preparation and make a drawing of a few of them.

Note the appearance of the cultures inoculated for the study of the gas production.

Read carefully in the text-books the methods for cultivating anaërobic bacteria.

EXERCISE XXIV

STUDY OF THE GAS PRODUCTION BY BACTERIA

92. The knowledge of the powers of a given species of bacteria to produce gas when grown in a medium containing sugar is quite important. It is desirable to determine both the quantity of gas and its relative composition. Chemical analyses have shown that, in all cases tested, the gas resulting from the fermentation of the sugar consists of a mixture of hydrogen (H) and carbonic acid gas (CO_2) with mere traces of other gases. It is important to know also the quantity of gas produced with the various sugars, especially glucose, lactose, and saccharose. To determine simply whether an organism will produce gas it is only necessary to inoculate it into tubes of liquid agar containing the various sugars; but if the quantity of gas is to be determined, the fermentation tube is the most convenient apparatus to use. In some cases the gas formation is one of the most striking differential properties, as will be seen in the study of hog-cholera and typhoid bacilli.

REFERENCES.—For a discussion of the gas production and use of the fermentation tube, see Smith, *Wilder Quarter-century Book*, 1893, p. 187; for the chemical formulæ, see Novy, *Laboratory Work in Bacteriology*, 1899.

93. Work for this Exercise.—Examine and describe the cultures in the glucose and plain agar inoculated in Exercise XXII. Note the approximate size and number of gas bubbles in the glucose agar, and explain the cause of difference in the number of bubbles in the two agar cultures.

Examine the fermentation tubes, and indicate the quantity of gas and the ratio of gas to liquid in the closed branch.

Determine the ratio of CO_2 to H in the gas.

In studying these cultures they should be examined each

day and the quantity of gas indicated. Note the bubbles of gas rising through the liquid to the top. When the gas production has ceased, the liquid begins to clear near the surface in the closed branch. The final record should not be made until this occurs. The reaction of the culture should be determined and noted at this and the next exercise. Explain the chemical formulæ for the production of the gas, and if the reaction changes, give the explanation. *to day*

Liquefy two large tubes of gelatin and two of agar, and pour the liquefied media from each tube into a Petri dish. After it sets, place the dishes on the table in the laboratory, and remove the covers. Allow one each of the dishes containing gelatin and agar to be exposed for 5 minutes and the others for 10 minutes. Label, and place the agar plates in the incubator and the gelatin ones in the cabinet for gelatin plates. Examine at the next exercise.

94. Determine the Quantity of Gas.—It is desirable to determine the quantity of gas collected in the closed branch in terms of the capacity of the tube. To do this, measure the length of the closed branch and the length of that portion of the tube filled with gas. Thus, if the length of the tube is 10 cm. and the length of the portion filled with gas is 3 cm., the gas fills three-tenths of the branch. This cannot be determined until the gas formation has ceased, which sometimes requires several (4 to 6) days. The closed branch of the fermentation tube should be straight and the connecting part of the tube should be narrow. If the tube stands too long before the quantity of gas is determined, some of it is liable to be absorbed.

95. To determine the Ratio of CO_2 to H in the Gas Produced.—This can be approximately determined by the use of caustic soda. Remove the plug from the fermentation tube and fill the open bulb with a 2% solution of caustic soda. Place the thumb tightly over the open end of the tube, and tip it up so that the gas will pass through the liquid and

come into the open bulb. It is then returned. This should be repeated several times. Remove the thumb when the open bulb is full, and the liquid will rush up into the closed branch to fill the space occupied by the CO_2 which has been absorbed by the caustic soda. Measure the portion of the tube first occupied with gas and now filled with the liquid. This will indicate the quantity of CO_2 . The remainder of the gas is H. (There are also traces of other gases.) Its explosive property can be tested by filling the open bulb with water, cover it with the thumb and again bring the gas to the open bulb, hold it close to a flame and remove the thumb. A distinct explosion will be heard.

The ratio of CO_2 to H can be determined from the measurements. Thus the total amount of gas = 5 cm. The amount absorbed (CO_2) = 2 cm. The remaining gas, or 3 cm., = the H. The ratio of CO_2 to H is, therefore, as 2 : 3 or $\text{CO}_2 : \text{H} :: 2 : 3$.

EXERCISE XXV

IDENTIFYING GENERA OF BACTERIA FROM THE DUST
IN THE AIR AND OBTAINING PURE CULTURES FROM
COLONIES

96. Work for this Exercise.— Examine and carefully describe the colonies on the plate cultures made by exposing agar and gelatin plates to the air in the last exercise. Determine the number of different colonies and carefully describe each. Explain the reason for variations in the number of colonies on the different plates.

Inoculate a tube of bouillon from a colony of each genus. Label the inoculated tube with the name of the genus. Make stained cover-glass preparations from a colony of each genus. Mount and label one of each of these preparations to accompany the notes.

EXERCISE XXVI

CLEANING USED CULTURE TUBES, FLASKS, AND PETRI
DISHES

97. Work for this Exercise. — Describe the bouillon cultures made from the colonies on the agar plates (Exercise XXV). Describe the bacteria in the stained cover-glass preparations and compare them with the bacteria in the preparations made from the colonies. State fully in the laboratory report the morphologic characters by which these genera are determined.

Reject and clean all tubes and Petri dishes that have been used for cultures. (See 3, *f*.)

EXERCISE XXVII

PREPARATION OF GLASSWARE FOR CULTURE MEDIA

98. It is important that the technique in plugging and sterilizing test tubes and flasks and in making culture media should be thoroughly understood. For this reason it seems desirable to again prepare and sterilize the glassware and to repeat the preparation of certain of the more commonly used culture media. Hereafter, in the course, the janitor will clean all glassware, and the instructor will furnish all additional media required. It will be necessary in the following exercises to overlap, to a considerable extent, by way of inoculating media and in making the final examinations of cultures the work of succeeding exercises.

99. Work for this Exercise. — Plug and sterilize the test tubes and flasks and sterilize the Petri dishes which have already been used and cleaned (Exercise XXVI).

EXERCISE XXVIII

PREPARATION OF CULTURE MEDIA

100. In this exercise some of the more specialized media used in determining certain of the physiologic and biochemic properties of bacteria are added. A few media required by certain pathogenic bacteria, either for their artificial cultivation or to aid in their differentiation, are also included. There are, however, many other media often referred to in the literature of this subject as being of more or less importance, but those mentioned in this exercise seem to be more commonly used. While it is not expected that the regular student will be able to prepare all of these media, it is essential that the methods of preparing them should be fully understood. If, however, any student desires, he has the privilege of making all of them. For convenience of reference the media are arranged in groups with the minimum number of tubes of each to be prepared.

101. Work for this Exercise. — Prepare after the methods already given (Exercises III., IV., and X.) the media in Group A.

102. Groups of Culture Media. — These are arbitrarily arranged for convenience of reference.

GROUP A. — (Media commonly used.)

- | | | |
|---------------|---|----------------|
| 10 | Small test tubes of bouillon. | } <i>omit.</i> |
| 10 | Small and 15 large test tubes of agar. | |
| 1 | (10) Small and (15) large test tubes of gelatin. | |
| 1 | (10) Small test tubes of milk. | |
| 1 | (10) Small test tubes of potato. | |

GROUP B. — (Media used to determine the power of bacteria to produce acids and alkalies.)

- | | |
|---|--|
| 1 | (10) Small test tubes of litmus milk. |
| 1 | (10) Small test tubes of sugar-free bouillon. |

- (5) Small test tubes of bouillon containing 1% grape sugar (dextrose, glucose).
- (5) Small test tubes of bouillon containing 1% milk sugar (lactose).
- (5) Small test tubes of bouillon containing 1% cane sugar (saccharose).

GROUP C. — (Media favorable for determining the production of gas.)

- (5) Small test tubes of agar containing 1% grape sugar.
- (5) Small test tubes of agar containing 1% milk sugar.
- (5) Small test tubes of agar containing 1% cane sugar.

GROUP D. — (Media and tubes favorable for approximate gas analysis and the determining of the aërobic or anaërobic tendencies.)

- (4) Fermentation tubes containing 1% grape sugar.
- (4) Fermentation tubes containing 1% milk sugar.
- (4) Fermentation tubes containing 1% cane sugar.

(The fermentation tubes containing bouillon with sugars may be substituted for the media containing sugars in Groups B and C if desired.)

GROUP E. — (Media either necessary for, or especially desirable for, the cultivation or differentiation of certain pathogenic bacteria.)

Acid agar (put in small test tubes).

Acid glycerin agar (put in small test tubes).

Acid glycerin bouillon (put in small test tubes).

Blood serum (dog) solidified at 70–75° C. (ground-glass-capped tubes).

Loeffler's blood serum (usually small test tubes).

103. The Preparation of Acid Agar. — This is prepared the same as ordinary agar (17) with the omission of the sodium hydrate in the bouillon from which it is made.

104. The Preparation of Acid Glycerin Agar. — Add 5% glycerin to acid agar before sterilizing it.

105. The Preparation of Acid Glycerin Bouillon. — This is prepared either as ordinary bouillon (12), or as sugar-free bouillon (59), with the omission of the alkali and the addition of 5% c. p. glycerin.

106. The Preparation of Blood Serum. — When a small quantity is sufficient, it can be obtained from a dog aseptically. The animal is properly tied on the operating table, etherized, the skin over the carotid or femoral artery is thoroughly disinfected and turned back, the artery exposed, a sterile glass canula inserted, and the blood collected in a sterile flask by means of a sterile rubber tube attached to the canula. After the serum is formed, it can be drawn off with a sterile pipette, and distributed in small sterile test tubes (5–7 c.c. in each). It is well to set the liquid serum in an incubator for a few days to test its sterility. The tubes of liquid serum are inclined (the same as agar) and placed in a blood serum sterilizer, or other chamber in which the temperature can be raised to 70° or 75° C., until the serum has set. Store in a cool place.

If larger quantities of the blood are required, it is more convenient to collect it from bleeding animals in a slaughter-house. In this case it is often necessary to sterilize the liquid serum after it has been distributed in tubes. This can be done in a water bath at 62° C. for 2 hours each day for four consecutive days.

107. The Preparation of Loeffler's Blood Serum. — This consists of 1 part neutral bouillon (prepared from meat), containing 1% grape sugar, 3 parts liquid blood serum. Mix and distribute in sterile test tubes, incline and solidify the same as blood serum. The temperature should be about 75° C., and the exposure will be necessarily longer than for the pure blood serum. When it is to be used for the cultivation of diphtheria organism, it can be set at a much higher temperature (80° to 100° C). Label and store.

For other methods and special media, see text-books.

EXERCISE XXIX

A STUDY OF CERTAIN PYOGENIC BACTERIA

108. There are a number of bacteria which are able to cause suppuration, but ordinarily the formation of pus is due to the presence of certain streptococci and micrococci. A number of bacilli, especially *B. coli communis* and *Ps. pyocyaneus*, are frequently found as the apparent cause of suppuration. As it is impossible to study more than a very few of these species, two of the most common and one more rarely encountered organism in suppurating wounds and abscesses are chosen for special study.

REFERENCES. — Chapters on pyogenic bacteria in text and reference books. Christman (recherches sur la suppuration), *Annals de l'Inst. Pasteur*, II. (1888), p. 469, Lucet (in animals). *Ibid.*, VII. (1893), p. 325; Von Lingelsheim (concerning streptococci), *Zeitschrift für Hygiene*, Bd. X. (1892), S. 331; Moore, *Am. Vet. Review*, Jan., Feb., and March, 1900.

109. Work for this Exercise. — Inoculate a tube of each medium in Groups A and B, from each of the cultures of the following bacteria which will be furnished. *Streptococcus pyogenes* and *Micrococcus pyogenes aureus*. *from abscesses*

Read carefully the chapter on pyogenic bacteria in the text-book.

Give in your laboratory notes a short abstract of one of the articles referred to above.

EXERCISE XXX

PYOGENIC BACTERIA (*Continued*)

110. Work for this Exercise. — Examine and carefully describe the cultures made in Exercise XXIX. Note especially the growth on the agar, gelatin, potato, and in the tubes of the bouillon containing the sugars. In describing the color, use color charts which are in the laboratory.

Examine microscopically in (1) hanging-drop and (2) stained (alkaline methylene-blue) cover-glass preparations the bacteria from each of the bouillon and agar cultures.

Measure a few of the bacteria in the stained preparations from the agar cultures, and make a drawing of them, magnified 1000 diameters.

Inoculate for Exercise XXXI. a tube of each medium in Groups A and B from a culture (furnished) of *Pseudomonas* (Bacillus) *pyocyaneus*.

For suggestions in studying cultures and microscopic preparations of bacteria, see Exercises VI. and XII.

111. Making Drawings of Bacteria with a Definite Magnification. — In measuring the bacteria we obtain the dimensions in microns or in units of $1/1000$ of a millimetre. In making a drawing, therefore, showing them magnified 1000 diameters, it is simply necessary to represent each micron by 1 millimetre. Thus, if the organism is 2.5μ in length and 1μ broad, the drawing should be 2.5 mm. long and 1 mm. broad. If the drawing is to represent the organism magnified 500 diameters, then each micron should be represented by 0.5 mm. For this purpose a metric rule and a pair of dividers are necessary.

EXERCISE XXXI

PSEUDOMONAS (BACILLUS) PYOCYANEUS

112. *Pseudomonas pyocyaneus*, commonly known as the bacillus of green pus, blue pus, or blue-green pus, is quite widely distributed in nature. While ordinarily it has been considered of little pathogenic importance, it is known to possess at times, and under certain conditions, marked infecting powers. This organism has been called the honey bacillus, on account of the peculiar odor emitted from its cultures. It is to be differentiated from *B. fluorescens liquefaciens* and its varieties which frequently appear in water.

REFERENCES. — Chapters on this organism in text-books. Barker, The Clinical Symptoms, etc. The Jour. of the Am. Med. Asso., July 31, 1897. Lartigau, Study of Pathogenesis, etc., Jour. of Exp. Med., 1898, p. 595. Jordan, Pigments produced by. *Ibid.*, 1899, p. 627. Růzicka, Arch. für Hygiene, Bd. XXXIV., S. 149, and Bd. XXXVII., S. 1.

113. Work for this Exercise. — Examine very carefully and describe fully the cultures of *Pseudomonas pyocyaneus* made during the last exercise.

Make and examine a hanging-drop and a stained cover-glass preparation from each of the bouillon and agar cultures.

Describe the appearance of the bacteria in each.

Measure and make a drawing of a few organisms in the preparation from the agar culture. Magnify 500 diameters.

Reëxamine the cultures of the streptococcus and the micrococcus studied at the last exercise and note all appreciable changes which have taken place.

Inoculate a tube of each of the media in Groups A and D, a tube of litmus milk, and one of sugar-free bouillon from a culture of *B. coli communis* (furnished), for study at the next exercise.

EXERCISE XXXII

BACILLUS COLI COMMUNIS

114. Of the bacteria normally present on the mucous membranes of the animal body the colon group is, on account of its close morphological relationship to the bacilli of typhoid fever and hog cholera, of more than ordinary interest. There are varieties of this organism which approximate very closely in their biochemic properties as well as in their morphology to the typhoid and also to the hog-cholera bacilli. It is important that this existing variation be recognized, and that the list of properties which characterize *B. coli communis* should be clearly determined. The differentiation of the colon and typhoid bacilli, as they exist in nature, is one of the difficult problems in practical bacteriological work. The culture assigned approaches very closely to the typical species.

REFERENCES.— Chapters on this organism in the text-books, T. Smith, *The Am. Jour. of Med. Sci.*, Sept., 1896. Adelaide W. Peckham, *Jour. of Exp. Med.*, Vol. II. (1897), p. 549. Adami, *Ibid.*, Vol. IV. (1899), p. 349.

115. Work for this Exercise.— Describe the appearance of each of the cultures of *B. coli communis* made in Exercise XXXI.

Examine the bacteria in a hanging-drop preparation from the bouillon and glucose bouillon cultures.

Make and stain with carbol fuchsin a cover-glass preparation from the agar culture. Measure a few of the bacilli and record their size in the notes.

Note especially the quantity of gas formed in each of the fermentation tubes. These cultures should be kept until the next exercise, when they should be examined again. If the gas formation is then completed, determine the quantity of gas and the ratio of the CO_2 to the H in the gas in each tube.

Make two gelatin plates from the bouillon culture. In making these plates use a tube of sterilized distilled water for the first dilution.

Test the culture in sugar-free bouillon for the presence of indol. Read chapter on this organism in text-book.

116. The Indol (Cholera Red) Test.—Add 1 c.c. of a .01% solution (fresh) of potassium nitrite and a few drops of concentrated sulphuric acid to the culture in sugar-free bouillon. A pinkish color indicates the presence of indol. In an old (3 to 5 day) culture the reaction is usually stronger than in a more recently made one.

If sugar-free bouillon is not at hand, a tube of *Dunham's solution* can be used instead with quite good results.

117. Dunham's Peptone Solution.—This is simply a solution of peptone and sodium chloride in distilled water. The formula is as follows:—

Dried peptone	1 gram.
Sodium chloride	0.5 gram.
Distilled water	100 c.c.

Dissolve the peptone and salt in the water and distribute it in the tubes (7 c.c. each) and sterilize the same as bouillon.

EXERCISE XXXIII

BACILLUS COLI COMMUNIS (*Continued*)

118. Work for this Exercise. — Reëxamine the cultures of *B. coli communis* and note any changes which have occurred in their appearance. Determine the gas formula in the fermentation tubes with the different sugars. Place the milk and litmus milk cultures in the incubator and examine later.

Examine and describe fully the colonies on the gelatin plates. Preserve the plates and examine them at the following exercises.

Examine microscopically, in a stained preparation, the bacteria from a colony on the gelatin plate. Preserve a preparation to accompany the notes.

Isolate *B. coli communis* from the intestine of an animal. The intestine will be furnished.

Inoculate, for Exercise XXXIV., a tube of each medium in Groups A and D and a tube of litmus milk and sugar-free bouillon with *B. cholerae suis*, and similar tubes with *B. typhosus*, from the cultures furnished.

119. Isolating B. Coli Communis from the Intestine. — Carefully open the intestine by a longitudinal incision. Scrape away the contents, if any, from a small area of the mucous membrane. Take a loopful of the mucus from the surface of the mucous membrane and inoculate a large tube of liquefied gelatin with it. After shaking the tube carefully, inoculate a second tube with 2 loopfuls from the first, and a third with 3 loopfuls from the second. Pour the gelatin into Petri dishes and label them. These plates should be examined daily. The colonies of *B. coli communis* can be distinguished from others which may appear by their thin spreading growth, sharply defined but irregular borders, and their bluish appearance, especially with transmitted light. Compare with colonies on gelatin plates from a pure culture, Exercise XXXII,

EXERCISE XXXIV

BACILLUS CHOLERÆ SUIS AND BACILLUS TYPHOSUS

120. The bacilli of typhoid fever and of hog cholera resemble each other very closely morphologically and in certain of their cultural characters and biochemic properties. Like *B. coli communis* each of these organisms has several varieties. Already several distinct varieties of the hog-cholera bacillus have been described. (The hog-cholera group of Bacteria. Bulletin No. 6, U. S. Bureau of Animal Industry, p. 9.) Certain of the varieties of these species approach each other very closely, while others approach *B. coli communis* in their various manifestations. It is important, therefore, that the morphology and properties of each of these species should be carefully determined. The fact should be kept clearly in mind that while these two species and the colon bacillus resemble each other in certain directions, they are, so far as has yet been demonstrated, distinct species. The special methods of differentiation must be omitted from this elementary course. Read carefully the chapter on *B. typhosus* in the text-book.

REFERENCES. — To hog cholera. Salmon, Special Report on Hog Cholera, Bureau of Animal Industry, U. S. Depart. of Agric., 1889. Smith, Bulletin No. 6. *Ibid.*, 1894. Moore, Report N. Y. State Commissioner of Agriculture, 1897. The Am. Vet. Review, March, 1898.

REFERENCES. — To typhoid. Chapters on this organism in text-books. Eberth, Virchow's Archiv, Bd. 81 (1880). *Ibid.*, Bd. 83 (1881). Gaffky, Mittheilungen aus d. Kais. Gesundheitsamte, II. (1884), S. 372. Lösener, Arbeiten aus d. Kais. Gesundheitsamte, XI. (1895). Péré, Annales de l'Inst. Pasteur, VI. (1882), p. 512. Jordan, Medical News, Sept. 28, 1895. Flexner, The Johns Hop. Hosp. Reports, Vol. V., p. 343. Smith, The Jour. of the Boston Soc. of Med. Sciences, June, 1898. Hiss, The Jour. of Exp. Med., Vol. II. (1887), p. 677.

121. Work for this Exercise. — Examine the plate cultures made from the intestine for the colon bacillus.

Determine the approximate number of colonies on each plate and note especially the number of colonies of *B. coli communis* and describe their appearance.

Inoculate a tube of agar, one of milk, and a fermentation tube of glucose bouillon from one of the colonies. Study these cultures in the next exercise and compare them with the notes on cultures of *B. coli communis* in these media.

Examine and carefully describe the cultures of *B. cholerae suis* and *B. typhosus*. Note especially the reaction of the cultures in the fermentation tubes. Examine the bouillon cultures microscopically (*a*) in hanging-drop and (*b*) in stained (methylene-blue) cover-glass preparations. Describe the appearance of the bacteria.

Make a series of 3 gelatin plate cultures from the bouillon culture of each organism.

EXERCISE XXXV

BACILLUS CHOLERÆ SUIIS AND BACILLUS TYPHOSUS

(Continued)

122. Work for this Exercise. — Reëxamine all of the cultures of *B. cholerae suis* and *B. typhosus*. Note especially the condition of the fermentation tubes. Keep the milk and the litmus milk cultures in the incubator for about 6 weeks and note any changes which may take place from week to week.

Examine and carefully describe the colonies on the gelatin plates.

Try the indol test (116) with the culture in sugar-free bouillon.

Make and stain with alkaline methylene-blue a few (3 or 4) cover-glass preparations from the organs (liver, spleen, kidney, or blood) of a rabbit which has died from the effect of the inoculation with hog-cholera bacilli. Note the number (few or many) of the bacteria in the preparations and preserve one of them to accompany the notes. Make a drawing of a few bacilli.

Examine and complete the notes on the culture of *B. coli communis*. Compare them with the cultures of hog-cholera and typhoid bacteria.

123. Making Cover-glass Preparations from Tissues. — With a pair of fine forceps take up a bit of tissue from the freshly cut liver, spleen, or kidney, and rub it gently over the surface of a clean cover-glass. Care must be taken that the film of tissue left on the cover-glass is thin. Allow this to dry in the air, after which pass the cover-glass, film up, three times through the flame to fix the tissue to the glass. They can be stained the same as the cover-glass preparations from the cultures. These are often spoken of as smear preparations.

In making these preparations from blood, hold a cover-glass

by the edge with a pair of dissecting forceps. Place a drop of blood with the platinum loop on the cover-glass near the forceps. Take a thick, square cover-glass by the edge, rest it on the first above the drop of blood, hold it at an angle of about 20° from it and draw it down over the first, thus spreading the blood in a very thin even film over the surface. If the film is thick, the preparation should be rejected and another one made.

EXERCISE XXXVI

BACILLUS CHOLERÆ SUIS AND BACILLUS TYPHOSUS

(Continued)

124. Work for this Exercise. — Reëxamine and complete the notes on all of the cultures except the milk and litmus milk, which should be kept for 3 weeks longer. Carefully observe the reaction of all the liquid cultures.

Stain the flagella on the bacteria from the agar cultures (82).

Compare the colonies on the gelatin plates with those of *B. coli communis*.

Make a careful comparison, in tabulated form, of the morphology, including measurements, of the bacilli themselves, and of the appearance of the growth in the different cultures of *B. coli communis* and the bacilli of hog cholera and typhoid fever.

The cultures, excepting those in milk, can be rejected now, or, if desired, they may be kept for further study and comparison.

EXERCISE XXXVII

WIDAL SERUM TEST

125. This test depends upon the fact that when the blood serum of a person suffering with typhoid fever, or who has recently recovered from it, is added to a bouillon culture of the bacillus, the bacilli become less motile and soon agglutinate in small clumps. The dilutions used vary from equal parts of serum and culture to dilutions of 1 to 50,000. It is recommended that the stronger dilutions shall be used, *i.e.* those from 1 : 10 to 1 : 50. The test has proven to be of much diagnostic value in typhoid fever.

It has been found that a similar reaction will take place with certain other bacteria when they are brought in contact with the serum from animals suffering from the disease which they produce. Thus it has been shown that such a reaction occurs with hog-cholera bacilli and serum from affected or immunized animals.

On account of the diagnostic value of this reaction it is employed very extensively in many health departments for the diagnosis of typhoid fever.

REFERENCES. — Black, Johns Hop. Hosp. Bulletin, Dec., 1896. Welch, The Jour. of the Am. Med. Asso., Aug. 14, 1897. Johnston, N. Y. Med. Jour., Oct. 31, 1896. Med. News, Jan. 23, 1896. Biggs and Park, The Am. Jour. of the Med. Sci., March, 1897. Westbrook and Wilson, The Phila. Med. Jour., March 26, 1898. Dawson, N. Y. Med. Jour., Feb. 20, 1897 (concerning hog cholera).

126. Work for this Exercise. — Take 1 loopful of a fresh bouillon culture of typhoid bacilli (which will be furnished) and place it on a cover-glass, add 1 loopful of blood serum from a typhoid patient or the blood of an immune guinea pig, and immediately make and examine a hanging-drop preparation with a loopful of the mixture. Note the effect on the

1 - 10 dilution.

motility of the bacilli and their aggregation into clumps. Specify the time elapsing before the agglutination appears and the time required for the complete clumping.

Make a similar examination of a culture to which 1/10 blood serum has been added.

Repeat the above test with the blood from animals affected with or immunized against hog cholera.

Examine a dried specimen of blood for this reaction. Add a drop of bouillon to the drop of dried blood on a slide, and after it has become well mixed add a loopful of it to a similar quantity of a fresh bouillon culture and examine it immediately in a hanging drop.

127. Securing Blood for the Widal Test. — (1) Dried preparations. Prick the finger or lobe of the ear (if a lower animal the shaved ear is a good place) sufficiently deep to procure a drop of blood. Place it on a slide by means of a platinum loop and allow it to dry. (2) Serum. From a similar but deeper prick, or by drawing a few drops of blood from a vein with a hypodermic syringe, secure a few drops of blood. Place them in the bottom of a small, short, sterile tube and allow the serum to ooze out. This can often be helped by separating the blood from the tube by means of a sterile wire. If retained for any length of time before making the test, the serum must be kept in a cool place. Experimentally, it is easily obtained by immunizing a guinea pig and then drawing the desired amount of blood from a vein.

EXERCISE XXXVIII

BACTERIUM (BACILLUS) SEPTICÆMIÆ HEMORRHAGICÆ
AND MICROCOCCUS LANCEOLATUS

128. These organisms are the causes of swine plague or infectious pneumonia in swine and of croupous or lobar pneumonia in man. *(non motiles)*

The name *Bacillus septicæmiæ hemorrhagicæ* was given by Hüppe to the bacillus of swine plague (Smith). This bacterium (bacillus) is morphologically and in its cultural characters not distinguishable from the bacterium (bacillus) of rabbit septicæmia (Koch), of fowl cholera (Pasteur), and of *Schweineseuche* (Schütz). It is similar to a species of pathogenic bacteria found more or less frequently in the upper air passages of nearly all of the domesticated animals. It is very similar also to a pathogenic bacillus found in broncho pneumonia in cattle and an infectious pneumonia in sheep.

Micrococcus lanceolatus is the specific organism of lobar pneumonia in man. It is found in the pneumonic lung tissue and also in the saliva of a certain number of healthy people. For the history and synonymy of this organism see article by Professor Welch in the Johns Hopkins Hospital Bulletin, Vol. III., p. 125. This organism resembles, in many of its properties, very closely the bacterium of swine plague. In studying the two species together there will be good opportunity of comparing them and detecting the differences and similarities existing between them.

REFERENCES.—To swine plague. Smith, Report on Swine Plague, Bureau of Animal Industry, U. S. Depart. of Agric., 1891. Smith, Zeitschrift f. Hygiene, Bd. X. (1891), S. 480. Smith and Moore, Bulletin No. 6. B. A. I., 1894. Moore, Report N. Y. State Com. of Agric., 1897.

REFERENCES. — To *M. lanceolatus*. Chapters on *Micrococcus lanceolatus* (diplococcus pneumoniae) in text-books. Welch, Johns Hop. Hosp. Bulletin, Vol. III. (1892), p. 125.

129. Work for this Exercise. — From the cultures furnished make a hanging-drop and a stained cover-glass preparation from each and inoculate a tube of each medium in the Groups A and B from each culture.

Examine carefully the hanging-drop preparations and describe the appearance of the bacteria in each.

Stain the cover-glass preparations with an aqueous solution of methyl-violet and carefully examine and describe the bacteria. Measure a few of them with the filar micrometer and make a drawing of a few organisms magnified 1000 diameters.

EXERCISE XXXIX

BACTERIUM (BACILLUS) SEPTICÆMIÆ HEMORRHAGICÆ
AND MICROCOCCUS LANCEOLATUS (*Continued*)

130. Work for this Exercise. — Carefully examine and describe the cultures made in Exercise XXXVIII.

Examine the agar and bouillon cultures microscopically in both the living condition and in cover-glass preparations stained with alkaline methylene-blue, carbol fuchsin, and an aqueous solution of methyl-violet.

Describe the appearance of the bacteria and make a drawing of a few of them from one preparation.

Preserve a preparation of each species to accompany notes.

If there is any growth in the gelatin tube, make a series of 3 gelatin plates from the bouillon culture.

EXERCISE XL

BACTERIUM (BACILLUS) SEPTICÆMIÆ HEMORRHAGICÆ
AND MICROCOCCUS LANCEOLATUS (Continued)

Has capsule

131. Work for this Exercise. — Reëxamine all the cultures of these bacteria, paying special attention to the reactions of the liquid cultures. *aqueous solution of fuchsin*

Make the indol test with the cultures in sugar-free bouillon.

Make, stain, and examine a cover-glass preparation from an organ or the blood of a rabbit which has died from the inoculation with swine-plague bacteria, and also a preparation made from a rabbit which has died from the inoculation with *Micrococcus lanceolatus*. Stain the preparations with an aqueous solution of fuchsin. Study the bacteria in these preparations and carefully compare the two. Indicate in the notes the differences, if any are found.

Give in the notes a list of characters and cultural properties of *Bact. septicæmiæ hemorrhagicæ* (swine plague) that are of differential value. In what properties does it differ from *B. cholerae suis*?

Keep the cultures until the next exercise and compare them again, after which they may be rejected.

EXERCISE XLI

BACTERIUM (BACILLUS) OF TUBERCULOSIS

132. The tubercle bacterium does not grow readily on the ordinary media. For its cultivation blood serum, glycerin agar, or bouillon containing 5 to 7% glycerin are ordinarily used. Formerly it was with much difficulty that it was made to grow from lesions in tuberculous animals; but when a culture was once started it could, on the media mentioned above, and sometimes on agar, be cultivated in subcultures with comparative ease. More recently Dr. Theobald Smith has described a method which renders its cultivation from tuberculous lesions much easier. (For details, see Appendix IV.) It grows very slowly and it is necessary that the temperature should be kept, without variation, at about 37° C. On account of these difficulties it is not practicable, in a general course, to cultivate this organism, but cultures on solid and liquid media will be furnished by the instructor for examination. It is important, however, to be able to recognize this organism in tissues and sputum, and consequently the following additional exercise in staining and studying it is given.

REFERENCES. — Chapters on this organism in text books. Smith, *Jour. of Exp. Med.*, 1898, p. 451. Moore, *Med. News*, May 14, 1892 (methods of staining). Nuttall, *Johns Hop. Hosp. Bulletin*, 1891, Vol. II., p. 67. Dorset, *Report Am. Public Health Asso.*, XXIV., p. 157.

133. Work for this Exercise. — Examine and carefully describe the appearance of the cultures of the tubercle bacterium on glycerin agar and in glycerin bouillon furnished.

Make 2 cover-glass preparations from the culture furnished for that purpose and stain them with carbol fuchsin (86).

Make 4 cover-glass preparations from tuberculous sputum and stain for tubercle bacteria. It is often desirable to counter

stain the specimens from sputa. Stain two of them by Gabbett's method, and two with carbol fuchsin and decolorize without counter staining. Make a few (2 or 3) cover-glass preparations from the liver or spleen of a guinea pig which has died from tuberculosis and stain them for tubercle bacteria. Stain one with carbol fuchsin and decolorize with sulphuric acid, and stain one by Gabbett's method.

Indicate in the notes the essential differences between human and bovine tubercle bacilli respecting (1) morphology, (2) cultural properties, and (3) pathogenesis. Do these forms differ from avian tubercle bacilli? If so, in what respects?

Measure the tubercle bacteria in one of the preparations and make a drawing showing a few of them magnified 1000 diameters. *Look on page 70*

134. Making Cover-glass Preparations from Sputum.—Select the little yellowish colored masses, if present, remove them by means of the fine forceps or platinum loop, and spread them on the cover-glass in a thin layer. If the sputum is homogeneous, make the preparations the same as from cultures, using a small loopful of the liquid. If the sputum is viscid, it is necessary to use the forceps to spread the film on the cover-glass. When dry, the films are fixed by passing the preparations through the flame, after which they are ready to be stained.

Instead of using cover-glasses, it is the practice in some laboratories to spread the sputum in a thin film over the central part of a slide; dry, fix, and stain as with the cover-glasses. The water is dried off by using filter or blotting paper, and the preparation examined without a cover-glass. The method is said to be easier and quicker than the other, and the cleaning of cover-glasses is saved.

135. Gabbett's Method of staining Tubercle Bacteria.

(1) The stain (carbol fuchsin) : —

Fuchsin	1 gram.
Absolute alcohol	10 c.c.
5% carbolic acid	100 c.c.

(2) The decolorizer and counter stain : —

Methylene-blue powder	2 grams.
10% sulphuric acid	100 c.c.

Stain the preparation with the first solution as described in (86), then rinse in water and stain 1 minute with the second solution, which decolorizes and counter stains at the same time, and again rinse in water. If the film has a bluish tint, it is ready for examination ; if not, it should be stained a little longer in the second solution. In these preparations the tubercle bacteria should appear as slender, more or less curved, rod-shaped bodies of a deep reddish color, while the surrounding tissue and other bacteria present are stained a more or less intense blue.

Sudan III. is reported by Dorset to be a very good differential stain for this organism. A saturated solution in 80% alcohol is used. It is reported to be effective in differentiating the tubercle organism from that of leprosy and from the smegma bacillus.

EXERCISE XLII

BACTERIUM (BACILLUS) MALLEI

136. This organism grows most characteristically on potato and somewhat feebly in the other media heretofore used. It develops readily on acid agar and in acid glycerin agar and acid glycerin bouillon. For this reason it is not inoculated into all of the media. In diagnosing glanders, it is customary to inoculate guinea pigs with the suspected material (see Appendix III). From the lesions in these animals, if the disease develops, pure cultures can usually be obtained. It can be identified by its morphologic and cultural characters.

REFERENCES. — Chapters on *Bacterium mallei* in text books. Smith, The Jour. of Comp. Med. and Vet. Archives, March, 1890. deSchweinitz and Dorset, Jour. of the Am. Chem. Soc., Vol. XVII. (1898). Finkelstein, Centralb. f. Bakteriologie u. Parasitenkunde, Bd. XI. (1892), S. 433.

137. Work for this Exercise. — Inoculate a tube of potato, one of agar, one of acid agar, one of acid glycerin agar, one of glucose agar, and one of bouillon from a culture furnished. (The special media here introduced will be furnished by the instructor.) *also acid glycerine Bouillon.*

Stain cover-glass preparations (furnished) made from the lesions in guinea pigs which were inoculated with this organism. Stain one with alkaline methylene-blue and one with carbol fuchsin. Note especially the morphology and the extent the organisms take the stain. *10-15 sec.*

EXERCISE XLIII

BACTERIUM (BACILLUS) MALLEI (*Continued*)

138. Work for this Exercise. — Examine and carefully describe all the cultures of *Bacterium mallei*.

Make 3 cover-glass preparations from the acid agar and from the bouillon cultures, and stain one of each with alkaline methylene-blue, one with carbol fuchsin, and one with an aqueous solution of methyl-violet. Describe the bacteria and make a drawing of a few of them. Preserve 1 preparation. Keep the cultures and reëxamine them at each of the 3 following exercises. Note especially the character and color of the growth on the potato.

Examine carefully the lesions produced by the inoculation of *Bacterium mallei* in a male guinea pig.

Inoculate a tube of liquid agar (Liborius' method, paragraph 89) and a fermentation tube of glucose bouillon from a culture of tetanus bacilli (furnished) and place the inoculated tubes in the incubator.

EXERCISE XLIV

BACILLUS TETANI

139. The bacillus of tetanus occurs in nature as a common inhabitant of the soil, at least it is found in the soil in certain localities. They are believed to be more numerous in certain places where manure has been thrown in abundance. This organism is anaërobic and consequently must be cultivated according to methods necessary for such bacteria (see paragraphs 89–90). In its effect upon the animal body it remains at the point of inoculation, the disease being produced by the toxin elaborated by the bacilli.

REFERENCES. — See text-books. Kitasato, Zeit. f. Hygiene. Bd. X., S. 267. Wesbrook, Jour. Path. and Bact., Vol. III., p. 70, Kanthack (morphology). *Ibid.*, Vol. IV., p. 452. Vaillard and Rouget (etiology). An. de l'Inst. Pasteur, T. VII., p. 755.

140. Work for this Exercise. — Carefully examine the 2 cultures made in the last exercise of tetanus bacilli and describe their appearance.

Make 2 cover-glass preparations from the liquid culture and stain them with carbol fuchsin. Examine them microscopically and describe their appearance. Make a drawing of a few bacilli magnified 1000 diameters. Keep these cultures until the next exercise, when they should be reëxamined and rejected.

Inoculate a tube of each medium in Groups A and B with *Bacterium anthracis* from a culture furnished for study at the next exercise.

141. Method of Isolating Tetanus Bacilli.—Tetanus bacilli rarely extend beyond the place of inoculation, into the body, of the infected individual (man or lower animal). In the local lesion there are almost always other bacteria, so that cul-

tures made directly from the lesions are usually impure. I have found that very often pure cultures may be obtained by inoculating a guinea pig with the pus or exudate from the local lesion and making cultures from the local lesions in the guinea pig, the juices of the body having destroyed the saprophytic bacteria which were present in the first material.

Kitasato has recommended a procedure which is reported to be fairly successful. It is to inoculate a tube of agar with tissue from the local lesion, and after it has grown for 24 to 48 hours at a temperature of 37° C., heat the tube to 80° C., which kills all the other bacteria, but does not destroy the tetanus spores. From this culture anaërobic cultures are prepared.

EXERCISE XLV

BACTERIUM (BACILLUS) ANTHRACIS

142. Work for this Exercise. — Examine and describe each of the cultures of this organism made during the last exercise.

Examine microscopically the bouillon and agar cultures in both hanging-drop and stained cover-glass preparations. Stain a preparation with each of the 3 ordinary staining solutions heretofore used.

Measure a few of the bacteria in a stained preparation and make a drawing of them magnified 1000 diameters.

Make a series of 3 agar plates from the bouillon culture.

Examine sections of animal tissue containing anthrax bacteria. Make and examine a few cover-glass preparations from the liver of an animal (guinea pig ~~or rabbit~~) which has just died of anthrax. (This will be furnished by the instructor.)

EXERCISE XLVI

BACTERIUM (BACILLUS) ANTHRACIS (*Continued*)

143. Work for this Exercise.—Reëxamine all of the cultures of *Bacterium anthracis* and describe any changes in their appearance which may have taken place.

Examine the agar culture for spores in (1) a hanging-drop preparation and (2) a stained cover-glass preparation. Describe the appearance of the bacteria and spores in a preparation from each.

Stain the spores (Exercise XIX.).

Study and describe the appearance of the colonies on the agar plates. Make an outline drawing of a few of the surface and deep colonies.

Reject all cultures except the agar plates, which may be kept until the next exercise for further observation before rejecting. (These cultures should be put in charge of the instructor, who will see that the spores are destroyed before the tubes are cleaned.)

Inoculate a tube of each medium in Group A and also a tube of Loeffler's blood serum with *Bacterium diphtheriæ* from a culture furnished for study at the next exercise.

REFERENCES.— Chapters on anthrax in text-books. Chester, Report Delaware College Agric., Exp. Station, July, 1895. Moore, Report N. Y. State Com. of Agr., 1897. Emmerich and Saida, Centralb. f. Bakter. u. Parasitenkunde, Bd. XXVII., S. 776.

EXERCISE XLVII

BACTERIUM (BACILLUS) DIPHTHERIÆ

144. The bacterium of diphtheria is often called the Klebs-Loeffler bacillus. It is the specific cause of diphtheria in man; but it is not, so far as known, the cause of diphtheria in pigeons and poultry. It is found in the throat of people suffering with diphtheria, and often in the throat and nose of those who have been exposed to the disease. These are designated as "germ cases." It is not found ordinarily elsewhere in the body, although it is occasionally discovered in the internal organs and blood. It usually remains in the throat for some days after its lesions have disappeared. Its appearance in the throat lesions is availed of in diagnosing the disease. For this reason it is especially important that its morphology, as well as its cultural characters, should be carefully determined. Although this organism grows on nearly all of the media commonly used, its development is more rapid and its growth more characteristic on Loeffler's blood serum. The bacterium of diphtheria seems to be modified in its morphology in growing on different media more than any of the other pathogenic bacteria. Particular attention should be given to its morphology and staining properties.

REFERENCES. — Chapters on diphtheria in text-books. Loeffler, *Mitth. aus d. Kais. Gesundheitsamte*, Bd. II. Biggs, Park and Beebe, *Scientific Bulletin No. 1*, Health Dept. City of New York, 1895. Wesbrook (varieties), *Jour. Boston Soc. Med. Sciences*, Vol. IV., p. 75. Report Am. Pub. Health Asso., 1899, p. 546. Hill (branching forms). *Ibid.*, p. 554. Bergey (pseudo-diphtheria), *Publications of the University of Pennsylvania*, new series, No. 4, 1898. Smith (toxin), *Trans. of the Asso. of Am. Phys.*, 1896. (Current medical literature contains many articles on this subject.)

145. Work for this Exercise. — Examine and describe the cultures made in Exercise XLVI.

Examine the agar and bouillon cultures microscopically in (1) hanging-drop preparations and (2) stained cover-glass preparations. Stain with alkaline methylene-blue and with carbol fuchsin. The preparation should be stained for fully 5 minutes with the alkaline methylene-blue. Also stain a preparation after Gram's method (38).

Examine carefully a fresh culture made directly from a diphtheritic throat, including stained cover-glass preparations.¹ Stain with alkaline methylene-blue and by Neisser's method. (It is not always possible to obtain these cultures at this particular time, in which case the examination will be postponed until they are available.)

146. Neisser's Method of Staining Diphtheria Bacteria. — Neisser has recently recommended the following method of staining, in which 2 solutions are employed, viz: —

(a) One gram of methylene-blue (Grübler's) is dissolved in 20 c.c. of 96% alcohol, which is then mixed with 950 c.c. of distilled water and 50 c.c. of glacial acetic acid.

(b) Two grams of Vesuvin are dissolved in 1 litre of boiling distilled water and filtered.

The cover-glass preparations are stained in (a) for 1 to 3 seconds, washed in water, and then stained in (b) for from 3 to 5 seconds, again washed in water, dried, and mounted. Stained in this manner the bacilli are brown, and contain 2, or rarely 3, but never more, blue corpuscles. The corpuscles are oval, not round, in shape, and their diameter appears greater than that of the bacilli in which they are situated.

¹ Clinically, *Bacterium diphtheriæ* is to be differentiated from the pseudo-diphtheriæ organism and from a bacillus which has been found in decayed teeth, and which is said to resemble in its morphology the Klebs-Loeffler bacillus very closely. It is also to be distinguished from the *Xerosis bacillus* isolated by Neisser. For detailed descriptions of these organisms see text-books.

EXERCISE XLVIII

BACTERIUM (BACILLUS) DIPHTHERIÆ (*Continued*)

147. Work for this Exercise. — Reëxamine all the cultures of *Bacterium diphtheriæ*, and describe all the changes which have appeared in them.

Examine microscopically, in stained cover-glass preparations, the bacteria from the glycerin agar and Loeffler's blood serum cultures. Stain with alkaline methylene-blue, and note especially the way the bacteria stain. Stain a few preparations after Neisser's method (the staining solutions will be furnished) and compare with the methylene-blue stain:

Note with special care the morphology of the bacteria and make a drawing of a few of them.

Examine sections of diphtheritic membrane showing diphtheria bacteria, and streptococci (furnished). Reject all cultures of diphtheria bacteria.

Examine very carefully a guinea pig (furnished) which has died from the effect of inoculation with diphtheria organisms.

Expectorate into a watch-glass which has been wiped with a cloth moistened in a 5% solution of carbolic acid. From this sputum inoculate a tube of bouillon and one of slant agar, and make a series of 2 agar and one of 2 gelatin plate cultures. Use a small loopful of sputum for each tube culture and the same for the first tube in the plate series.

EXERCISE XLIX

THE BACTERIA OF THE MOUTH

148. In studying cultures from the throat of diphtheritic individuals one encounters many variations in the species of bacteria other than those of diphtheria which are present. The same condition holds true with the microscopic examination of sputum for the tubercle bacteria. The fact has been determined that the organism of lobar pneumonia is often found in the human saliva, and, furthermore, the bacterium of swine plague (*Bacterium septicæmiæ hemorrhagicæ*) is often in the upper air passages of a large percentage of healthy swine and a like organism in cattle, cats, and dogs. In order, however, to isolate them, it is usually necessary to resort to rabbit inoculation.

Much attention has been given to the study of the bacteria of the mouth, and it seems desirable that a few examinations should be made for the purpose of learning something definite concerning the variety of species which are normal inhabitants of, and which seem to be somewhat localized in, the oral cavity, and consequently which may be encountered in seeking for pathogenic forms. In addition to those forms, which seem to be more or less localized on the mucosa of the mouth, there is usually present in the oral cavity a large and changing variety of bacteria which have been introduced with the food.

REFERENCES. — Vicentini, Bacteria of the Sputa, London, 1897. Miller, Die Mikro-organismens des Mundhöle, Leipsic, 1889. David, Les Microbes de la Bouche, Paris, 1890.

149. Work for this Exercise. — Examine carefully and describe fully the cultures made from sputum at the last exercise.

Make a hanging-drop preparation from one of each of the different kinds of colonies and describe the appearance of the

organism. Note the name of the genus to which each colony belongs.

Give a summary of the genera of bacteria present on the plates with the approximate number of colonies of each.

Make 1 or more cover-glass preparations from the mouth and stain with alkaline methylene-blue. Note carefully the varieties of bacteria it contains.

Inoculate from the unnamed cultures furnished such media as the requirements of the next exercise demand.

150. Making Cover-glass Preparations from the Mouth.— These can be made from the sputum, expectorated in a watch-glass, or from the scrapings from the tongue, gums, pharynx, or from the base of the teeth. If any of the latter sources are chosen, the part from which the material is to be taken should be scraped carefully with a sterile (flamed) platinum loop or with the blunt point of a scalpel or other stiff instrument. The scrapings are spread on cover-glasses the same as the sputum.

EXERCISE L

DETERMINING THE SPECIES OF BACTERIA

151. The 2 cultures of bacteria assigned for identification belong to species already studied. The student should identify the species of bacteria in the cultures. To do this such media should be inoculated and such microscopic examinations made as he thinks necessary. The notes should contain a complete record of the work and the reasons for the identifications made.

152. Work for this Exercise.—Identify the bacteria in the cultures assigned at the last exercise. Use any method which seems to be necessary. In the laboratory notes give reasons for the procedure adopted.

EXERCISE LI

ISOLATING AND IDENTIFYING BACTERIA FROM ANIMAL
TISSUES

153. In making a bacteriologic investigation into the cause of death in an animal or man, it is necessary to make cultures from the various organs and the blood to find whether or not there are any pathogenic or other bacteria present. This necessitates a knowledge of making cultures from animal tissues. In this exercise an experimental animal (rabbit or guinea pig) will be provided which has died from some bacterial disease. The purpose of this examination is to find out what that disease is. To save animals, each student will make cultures from but one organ. Opportunity will be afforded from time to time during the course for making cultures from variously diseased animal tissues. For methods of inoculating animals for purposes of diagnosis, see Appendix III.

154. Work for this Exercise. — The experimental animal will be furnished tied out on a post-mortem tray and the viscera exposed. (Directions for the post-mortem examination will be given in the course in pathology.)

Inoculate a tube of bouillon, one of agar, and a fermentation tube of glucose bouillon from either the liver, spleen, or kidney. (In an actual investigation of an unknown disease, cultures should be made from all of the organs, blood, and lymphatic glands.)

Make a series of 3 agar plate cultures from the same organ.

Make several cover-glass preparations from the organ from which the cultures were made.

Stain and examine the cover-glass preparations and describe the bacteria, if any are found. Stain with alkaline methylene-blue and carbol fuchsin. (It is sometimes necessary to fix

pieces of the tissue in alcohol or in some other fixing fluid for sectioning and staining, preparatory to studying them.)

Preserve one or more of the cover-glass preparations to accompany the notes.

155. Making Cultures from Animal Tissues.— Heat a platinum spatula to a red heat in a gas flame and scorch the surface of the organ. Flame a pair of fine forceps and tear an opening through the scorched surface and crush a bit of tissue underneath it. With the platinum loop take up a loopful of the crushed tissue with which inoculate the media. It is also desirable to inoculate a tube of slant agar with the needle by drawing it over the surface of the medium after charging it with tissue. In making plate cultures use a loopful of the crushed tissue for the first tube. The quantity of the tissue necessary to give a desired number of colonies cannot be anticipated, although experience in working with different organisms in animals renders one able to approximate the amount required.

EXERCISE LII

ISOLATING AND IDENTIFYING BACTERIA FROM ANIMAL
TISSUES (*Continued*)

156. Work for this Exercise. — Examine and describe all of the cultures made from the animal tissues.

Examine the bouillon and agar cultures microscopically in the fresh condition and in stained cover-glass preparations.

If the species cannot be determined from these cultures and examinations, make such other cultures from these as may be necessary to enable one to do so. Examine these at the next exercise when the notes can be completed.

State in the notes the facts upon which the identification is based.

EXERCISE LIII

THE EXAMINATION OF SECTIONS OF TISSUE CONTAINING
BACTERIA

157. The preparation of tissues for sectioning and the study of the tissue changes more properly belong to the course in pathology. It is important, however, that one be able to distinguish bacteria in the lesions which they produce. For this reason an exercise is devoted to the study of bacteria in sections of tissues already stained and mounted. These include the various pneumonias, tuberculosis, anthrax, hog cholera, typhoid, septicæmia, etc.

158. Work for this Exercise.—Examine the sections furnished for bacteria and note especially their distribution in the tissues. Make drawings of a few of the bacteria from each preparation.

Compare the bacteria in the sections with the cover-glass preparations which have been made from cultures and note any differences in their appearance which may be detected.

EXERCISE LIV

BACTERIOLOGIC EXAMINATION OF PUS AND EXUDATES

159. It is often very desirable for diagnostic purposes to make a bacteriologic examination of the pus from abscesses and the muco-purulent discharges or exudates from mucous or serous membranes.

Several diseases can be diagnosed in this way. It is often necessary to make cultures and it is always advisable to do so whenever the material is in a suitable condition. Among the specific diseases for which such an examination is especially valuable are actinomycosis, gonorrhœa, diphtheria, and tuberculosis. Further, it is often desirable to determine the genera of the bacteria in the numerous abscesses and suppurating wounds encountered in both man and the lower animals. Such examinations will be made from time to time of the more desirable cases as they become available. In this exercise such cover-glass preparations will be examined as have been accumulated for this purpose.

160. Work for this Exercise.—Examine the pus in the fresh condition and note its composition, leucocytes, red blood corpuscles, fungi (actinomycosis), etc.

Make cover-glass preparations and stain one or more of them with carbol fuchsin and one with alkaline methylene-blue and examine. Note the cellular tissue elements present and describe the bacteria found. If the pus is from a case suspected to be of a specific nature, stain and examine for the corresponding organism.

If actinomycosis, the ray fungus may be seen better in the fresh preparation. Add a drop of a 10% solution of caustic potash to a loopful of pus on the slide and cover it with a cover-glass and examine.

If gonorrhœal discharge, stain the cover-glass preparations

with alkaline methylene-blue or with carbol fuchsin. Note the appearance of the cocci both within and outside of the pus cells.

If from supposed tuberculosis, stain for that organism.

If from diphtheria, stain for that organism and note the morphology of the bacteria.

If from the pus of an abscess, stain for pyogenic bacteria.

161. Making Cover-glass Preparations from Pus.—Spread as thin a film of the pus as possible on the cover-glass. This can be readily done by drawing the edge of a square cover-glass over the surface of another cover-glass on which a bit of the pus has been placed. See method for making cover-glass preparations from blood (123).

EXERCISE LV

A BACTERIOLOGIC EXAMINATION OF THE SKIN FOR MICROCOCCUS EPIDERMIDIS ALBUS AND OTHER BACTERIA

162. There is liable to be on or in the skin a number of bacteria which resist the ordinary methods of cleansing, owing to their being deeply seated in the epidermis. The most important among these is *M. (Staph.) epidermidis albus*. These organisms often infect wounds in surgical operations. An abrasion of the skin with a sterile instrument may be followed by the infection of the wound with this or other species of bacteria which were on or in the skin itself. The work of this exercise is to demonstrate the presence of these organisms on the skin of supposedly sterilized hands.

REFERENCES.—Dennis' System of Surgery, Vol. I., p. 249. This chapter, written by Professor Welch, contains a summary of the present knowledge of the bacteria of the skin, with references to original articles.

163. Work for this Exercise.—Wash the hands thoroughly with soap and water, using the brush. Then wash them in a solution of 1 to 1000 corrosive sublimate and rinse thoroughly in boiled water and wipe with a sterilized towel (furnished). State how the towels can be sterilized.

With a flamed and cooled scalpel scrape the epidermis over a small area about the finger nails and with these scrapings inoculate a tube of bouillon and make a series of 2 agar plate cultures.

Make a similar series of cultures with the scrapings from the back or palm of the hand.

At the next exercise describe these cultures and examine the colonies microscopically to determine the genera of bac-

teria. If a micrococcus which grows in clumps is found, inoculate a tube of agar with it and at the following exercise examine and describe its appearance. Indicate in the notes the number of colonies of bacteria which developed in the plate cultures and the genera which appear in the bouillon culture.

(EXERCISE LVI)

DETERMINING THE THERMAL DEATH POINT OF BACTERIA

164. It is important to know the minimum temperature which will kill bacteria, especially the pathogenic forms. The uses to which such knowledge can be put are numerous in practical sanitary medicine, disinfection, and pasteurization. For the various methods employed in making these determinations, see text-books and special articles on this subject. The method here given, and which can be followed by a full section of students, will give only approximate results. It should not vary, however, more than one degree from the actual thermal death point in moist heat of the organisms tested.

165. Work for this Exercise.—Inoculate 5 tubes of bouillon from each of 2 cultures (*B. subtilis*, old culture, and *B. typhosus*) furnished.

In inoculating be sure not to touch the sides of the tube with the inoculating loop. The tubes should have stood in the water bath at 60° C. for at least 15 minutes before they are inoculated.

Place one of these tubes in the incubator for a control. Stand the others in a wire basket and set them in the thermo-regulated water bath adjusted at 60° C. The water should come just above the liquid in the tubes. Remove the tubes, one of each species, as follows: one in 5 minutes, one in 10 minutes, one in 15 minutes, and one in 20 minutes. Label and place them in the incubator.

At the next exercise examine the heated tubes and note which are clear and which contain a growth. If the tubes heated for 10 minutes or longer have a growth, repeat the experiment at 70° C. If this fails to destroy them, repeat at 80° C., and if necessary apply a still higher temperature.

Examine the cultures microscopically in all the fertile tube to determine if they are pure.

Explain the cause for the difference in the thermal death point between these two organisms.

Inoculate for the next exercise a tube of bouillon from a culture of *M. (Staph.) pyogenes aureus* and one from a culture of *B. subtilis* furnished.

EXERCISE LVII

DETERMINING THE EFFICIENCY OF DISINFECTANTS

166. The efficiency of the more commonly used disinfectants has been determined for most of the pathogenic bacteria, but new disinfectants are constantly being put upon the market, and before it is safe to use or recommend them their efficiency should be determined. With many of the disinfectants, such as carbolic acid, corrosive sublimate, lime, and the mineral acids, much stronger solutions are commonly used than are actually necessary to kill the bacteria, owing to the fact that frequently it is necessary to allow for an indefinite waste due to the union of the disinfectant with other substances, usually organic, with which the bacteria are mixed. For the different methods of testing the efficiency of disinfectants, see text-books. A very simple process is given here.

It may be desirable for students to work in groups of two or more in order to economize in the number of tubes required in this exercise. If possible, however, each student should make all of the tests.

REFERENCES. — Young, Notes on Disinfectants and Disinfection, Augusta, 1898. Rideal, Disinfection and Disinfectants, London. See also text-books.

167. Work for this Exercise.—Prepare 20 c.c. of each of a .25 and .10% solution of formalin (40% formaldehyde in water), and distribute them in sterile tubes, putting 10 c.c. in each. Use distilled sterilized water in making the dilutions. Add, by means of a sterilized pipette, to each of the tubes in one set $\frac{1}{4}$ c.c. of a bouillon culture of *B. cholerae suis* or of *B. typhosus*. Add to each of the tubes in the other set the same quantity of the culture of *B. subtilis*.

Use a sterile pipette for adding the culture to the disinfectant.

Inoculate a tube of bouillon, containing fully 7 c.c., with 6 loopfuls from each of these tubes, after the expiration of the following periods of time: 1 minute, 5 minutes, 10 minutes, and 30 minutes. In making these inoculations allow the loop to go to the bottom of the inoculated tube. Label each inoculated tube with the strength of the disinfectant and time of exposure and place it in the incubator. It should be noted that the adding of $\frac{1}{4}$ c.c. of culture diluted slightly the strength of the disinfectant.

Note at the next exercise the condition of each inoculated tube. From them the approximate strength and time for the disinfectant to destroy the bacteria can be determined. When this is found the more exact strength and time can be determined by repeating the experiment with weaker dilutions or shorter exposures or both.

EXERCISE LVIII

TESTING DISINFECTANTS (*Continued*)

168. Work for this Exercise. — Prepare two sets of tubes, each containing 10 c.c. of a 2.5, 1.0, and 0.5% solution, respectively, of carbolic acid and test their effect upon the organisms used in the last exercise and by the same method. Note the conditions of the inoculated tubes at the next exercise.

Examine and describe the appearance of the tubes inoculated during the preceding exercise.

Allow the inoculated tubes to remain in the incubator for several days and note whether or not any of them develop after the first 24 hours. If they do, examine them microscopically to determine if the culture is pure. Observe in these cultures the difference between immediate destruction and the retarding of the growth of the bacteria.

EXERCISE LIX

PASTEURIZING AND STERILIZING MILK

169. Milk is pasteurized, in the present acceptance of the term, when all of the pathogenic bacteria which it may happen to contain (with the exception of the spores of anthrax) are destroyed, with the more important saprophytes. It is not necessarily sterile, although it sometimes is. The temperature and time for heating is from 60–68° C. for 20 minutes.

In this exercise it is the purpose to study the effect of this process on the bacteria of milk and to compare its effect with that of sterilization.

In the generally accepted use of the term, milk is sterilized when it has been boiled. Milk, however, is a difficult substance to sterilize, so that it occasionally happens that milk which has been boiled for from 5 to 10 minutes still contains living organisms (spores).

170. Work for this Exercise. — From the fresh milk provided make 3 agar plates, using 1, 2, and 3 loopfuls, respectively, of the milk. Put 25 c.c. in each of 2 large test tubes and set one in the incubator and leave the other at room temperature. Put 25 c.c. in each of 4 large test tubes. Sterilize two of them by boiling for 30 minutes in a closed water bath, and pasteurize the other two by heating them in the water bath for 30 minutes at 65° C. It requires about 10 minutes for the milk in the tubes to reach the temperature of the water, leaving the milk exposed to the temperature of the water for 20 minutes. It should be cooled quickly by standing the tubes in cold water.

After the tubes are cooled, make 3 agar plates from one of the tubes treated by each process, using 1 loopful of milk for the first plate, 3 loopfuls for the second, and $\frac{1}{4}$ c.c. (measure with a graduated pipette) for the third. Place one of the tubes of milk treated by each process with the plate cultures, in the

incubator, and leave the other tubes with a tube of the fresh milk at the room temperature.

At the next exercise note carefully the condition of the milk in each of the various tubes and also the number of colonies on the agar plates.

Keep the tubes of milk for further examination at the following exercise, noting any changes in their appearance, after which they may be rejected.

EXERCISE LX

THE QUANTITATIVE BACTERIOLOGIC EXAMINATION OF WATER

171. This is to determine the number of bacteria in water. In preparing media for this purpose the directions given in the Journal of the American Public Health Association for Jan., 1898, p. 60, should be followed. The conditions of temperature and of media which favor growth differ for different species. Many water bacteria will not grow at the incubator temperature, while others which may be in it grow very slowly at the room temperature. To determine numbers, it is better to grow the bacteria in gelatin plates at the temperature of the room. (In an actual examination a much larger number of plate cultures should be made than can be managed here.)

172. Work for this Exercise.— Make from the properly collected water 4 gelatin plates, using a definite quantity of water for each. To begin with, it may be safe to inoculate these tubes with 0.1, 0.25, 0.50, and 1.00 c.c., respectively.

To determine if there are gas-producing bacteria, and the approximate number of these if any, inoculate ~~2~~ fermentation tubes with 0.1 c.c. each and 5 with $\frac{1}{5}$ c.c. each. In place of the fermentation tubes glucose agar may be used. In this case 1 fermentation tube of glucose bouillon should be inoculated with 3 c.c. of the water to determine the quantity of gas produced if there is any. If a large fermentation tube is used, add 5 c.c. of the water. From the gas produced in these tubes determine approximately the number of the gas-producing bacteria.

Careful and full notes should be taken on this examination. The preliminary methods for making a bacteriologic examination have already been given and this is largely in the nature of an investigation by each student. It is not expected that

the special methods will be tried other than those used in the laboratory for pathogenic bacteria.

173. Collecting Water.—If the water is collected from a spigot or pump, allow it to flow for 2 or 3 minutes first, and then collect the desired quantity, 100–200 c.c., in a sterile bottle and cork tightly; or, if near at hand, absorbent cotton plugs may be used.

If from a stream or river, withdraw the stopper and immerse the sterile bottle, mouth downward, to the depth desired and allow it to fill. There are several mechanical devices for collecting water from considerable depths from the surface.

EXERCISE LXI

THE QUANTITATIVE EXAMINATION OF WATER (*Continued*)

174. Work for this Exercise. — Examine the cultures and count the colonies on the plates, and estimate from them the number of bacteria in a cubic centimetre of the water; that is, if there are 40 colonies on the plate containing .1 c.c. of water, there are 400 bacteria in 1 c.c. of it.

From the cultures in the grape-sugar media estimate the number of gas-producing bacteria present.

Describe the appearance of the different colonies and indicate the approximate number of each kind.

Keep the plate cultures until the following exercise and re-examine and count the colonies.

Determine the obviously different genera of bacteria by making a microscopic examination of the different colonies.

175. Estimating the Number of Gas-producing Bacteria in Water. — If there is gas in all of the 10 fermentation tubes inoculated with 0.1 c.c. each, it would show that there were 10 or more of these bacteria in each cubic centimetre. If 3 of the 5 tubes inoculated with $\frac{1}{5}$ c.c. each contained gas, it would indicate that there were at least 3 gas-producing bacteria in 1 c.c. The preliminary results must be verified by repeated examinations.

EXERCISE LXII

THE QUALITATIVE EXAMINATION OF WATER

176. The qualitative examination of water consists in determining the species of bacteria present. From a sanitary standpoint it consists in finding, if present, those species which may be the cause of disease among people or animals consuming it. The pathogenic bacteria which may be in the water will depend upon the conditions ; but usually in this country water is examined for typhoid and hog-cholera bacilli, *B. coli communis* and *Ps. pyocyaneus*.

In India the spirillum of Asiatic cholera may be found in the water. Occasionally anthrax may be suspected. It should be stated that *B. fluorescens liquefaciens*, pseudo-typhoid, and the transitional forms of the colon group are to be carefully differentiated from *Ps. pyocyaneus* and *B. typhosus*. Owing to imperfect descriptions many of the common soil and water bacteria cannot be readily identified. The genera are all that is expected here.

REFERENCES. — Frankland, Micro-organisms in Water. Fuller, Report Am. Public Health Asso., 1899, p. 580.

177. Work for this Exercise. — Make at least 4 gelatin plate cultures and such others as may seem necessary to determine the different species, especially of pathogenic bacteria and their relative number in the specimen of water furnished.

Read carefully the methods for water analysis in the textbooks.

EXERCISE LXIII

THE QUALITATIVE EXAMINATION OF WATER (*Continued*)

178. Work for this Exercise.— Examine the cultures made in Exercise LXII., and make such others (subcultures from colonies, etc.) as may seem necessary to determine the genera and species of bacteria present with the relative number of each per cubic centimetre.

EXERCISE LXIV

EXAMINATION OF CERTAIN BACTERIA NOT STUDIED IN
THE LABORATORY

179. This exercise will be devoted to a study of preparations of important bacteria, fungi, and pathogenic protozoa not cultivated in the laboratory. Unfortunately the number necessarily omitted is large. This demonstration, however, will aid in fixing in the mind an idea of the morphology of these forms which may be of some assistance. Certain of the pathogenic protozoa, such as the cause of Texas fever in cattle and malaria in man, will also be demonstrated. These will be studied more thoroughly in the course in Pathology.

180. Work for this Exercise.—Examine and make drawings of the bacteria in the preparations furnished.

Complete and hand in all notes on laboratory work.

Have all apparatus for individual use inspected by the instructor and returned to the laboratory.

APPENDIX

I

REACTION OF CULTURE MEDIA

THE importance of the reaction of media as a controlling factor in the development of biological characters is of so much importance that the methods recommended by the committee of bacteriologists appointed in 1895 to the American Public Health Association in 1897 are appended to aid those who may not have the transactions of that association at hand.

“The first thing to obtain is a standard ‘indicator’ which will give uniform results. These requirements are best fulfilled by phenolphthalein.

This indicator was first suggested by Schultze in combination with the titration method for obtaining the desired reaction for culture media (*Cent. für Bakt. und Parasit.*, Bd. X., 1891, S. 53), but its general adoption seems to have been retarded largely by Dahmen (*Cent. für Bakt. und Parasit.*, Bd. XII., 1892, S. 620), who claimed that its use was not feasible, owing to complications which might arise from the presence of carbonates and ammonium salts in the solution to be tested. These objections to the use of phenolphthalein do exist, but may be readily overcome.

The amount of free and combined ammonia present in culture media at the time the reaction is determined, has been found not to exceed .003%, which is less than one-tenth the amount which interferes with the accuracy of this indicator; while the production of carbon dioxide is obviated to a very great degree by neutralizing with sodium hydroxid instead of with sodium carbonate, and any of this gas which may be absorbed from the atmosphere is practically all driven off by heat during the preparation of the media.

The great advantage in the use of phenolphthalein over other indicators lies in the fact that it takes into account the reaction of

weak organic acids and of organic compounds which have an amphoteric reaction, but in which the acid character predominates. Turmeric possesses the same properties, but the change in color from a yellow to brown is less satisfactory than the development of purple red color, and furthermore turmeric paper changes color rather slowly, while with phenolphthalein the color appears almost instantly.

Another advantage to be gained from the use of this later indicator is its behavior toward the phosphates. Petri and Maassen (*Arbeiten aus dem K. Gesundheitsamte*, Bd. VIII., 1893, S. 311) and Timpe (*Cent. für Bakt. und Parasit.*, Bd. XIV., 1893, S. 845; Bd. XV., 1894, S. 394-664; Bd. XVII., 1893, S. 416) have shown that the amphoteric reaction of media is associated with the presence of phosphates, and that there are present in peptone and gelatin proteid bodies which possess both an acid and a basic nature, but in which the acid character predominates. These observers agree that to determine accurately the reaction of such amphoteric compounds phenolphthalein, or turmeric paper, should be used as an indicator.

It is known that at the neutral point of phenolphthalein any free phosphoric acid present enters into combination, and the monobasic and tribasic salts of this acid are changed to the dibasic form (Na_2HPO_4). Now disodium hydrogen phosphate reacts alkaline to litmus, lacmoid, rosolic acid, and methyl-orange, but neutral to phenolphthalein and turmeric.

Studies made at the Lawrence Experiment Station show that this acid salt may be added to culture media in amounts greatly exceeding those naturally present in the media without producing any apparent influence upon bacterial development.

From these facts it seems clear that the use of any of the above-mentioned indicators, other than phenolphthalein and turmeric, in the presence of this dibasic phosphate, prevents the addition of a sufficient amount of free alkali to effect neutralization, and as the amount of phosphates in media varies considerably, the reaction passes beyond accurate control when litmus and other substances of its class are used as indicators.

Datum point to which all degrees of reaction shall be referred:

From the available evidence it seems advisable to adopt the phenolphthalein neutral point as the fixed point to which all degrees of reaction shall be referred.

The question of the proper reaction of media for the cultivation of bacteria, and the method of obtaining this reaction, have been discussed in a valuable paper by Mr. George W. Fuller, published in the Journal of the American Public Health Association, Vol. 20, Oct., 1895, p. 321. Some of the main results there given have been mentioned above.

Method of determining the Degree of Reaction of Culture Media.—

For this most important part in the preparation of culture media, burettes, graduated into $\frac{1}{10}$ c.c., and 3 solutions are required.

1. A .5 % solution of commercial phenolphthalein in 50 % alcohol.
2. A $n/20$ solution of sodium hydroxid.
3. A $n/20$ solution of hydric chlorid.

Solutions Nos. 2 and 3 must be accurately made up and must correspond with the normal solutions soon to be referred to. Solutions of sodium hydroxid are prone to deterioration from the absorption of carbon dioxid and the consequent formation of sodium carbonate. To prevent as much as possible this change, it is well to place in the bottle containing the stock solution a small amount of calcium hydroxid, while the air entering the burettes or the supply bottles should be made to pass through a "U" tube containing caustic soda, to extract from it the carbon dioxid.

The medium to be tested, all ingredients being dissolved, is brought to the prescribed volume by the addition of distilled water to replace that lost by boiling, and after being thoroughly stirred, 5 c.c. are transferred to a 6-inch porcelain evaporating dish; to this 45 c.c. of distilled water are added, and the 50 c.c. of fluid are boiled for 3 minutes over a flame. One c.c. of the solution of phenolphthalein (No. 1) is then added, and by titration with the required reagent (No. 2 or 3) the reaction is determined. In the majority of instances the reaction will be found to be so that the $n/20$ sodium hydroxid is the reagent most frequently required. This determination should be made not less than three times, and the average of the results obtained taken as the degree of reaction.

One of the most difficult things to determine in this process is exactly when the neutral point is reached, as shown by the color developed, and to be able in every instance to obtain the same shade of color. To aid in this regard, it may here be remarked that in bright daylight the first change that can be seen on the

addition of alkali is a very faint darkening of the fluid, which on the addition of more alkali becomes a more evident color, and develops into what may be described as an Italian pink. A still further addition of alkali suddenly develops a clear and bright pink color, and this is the reaction always to be obtained.

All titrations should be made quickly and in hot solutions, to avoid complications arising from the presence of carbon dioxide.

When this manipulation is carried out uniformly, as here suggested, and the end point having the same intensity of color is always reached, very satisfactory and closely-agreeing results may be obtained.

Neutralization of Media. — The next step in the process is to add to the bulk of the medium the calculated amount of reagent, either alkali or acid, as may be determined. For the purpose of neutralization a normal solution of sodium hydroxid or of hydric chlorid is used, and after being thoroughly stirred the fluid thus neutralized is again tested in the same manner as at first to insure the proper reaction of the medium being attained. When neutralization is to be effected by the addition of alkali, it not infrequently happens that after the calculated amount of normal solution of sodium hydroxid has been added the second test by titration will show that the medium is still acid to phenolphthalein, to the extent sometimes of from 0.5 to 1 %. This discrepancy is perhaps due to side reactions, which are not understood; the reaction of the medium, however, must be brought to the desired point by the further addition of sodium hydroxid, and the titrations and additions of alkali must be repeated until the medium has the desired reaction (*i.e.* 0.0 % — 0.005 %, see below).

After the prescribed period of heating it is frequently found that the medium is again slightly acid, usually about 0.5 %. Without correcting this the fluid is to be filtered and the calculated amount of acid or alkali is to be added to change the reaction to the one desired.

A still further change in reaction is not infrequently to be observed after sterilization, the degree of acidity varying apparently with the composition of the media and the degree and continuance of the heat.

Manner of expressing the Degree of Reaction of Culture Media. — Since at the time the reaction is first determined culture media

are more often acid than alkaline, it is proposed that acid media be designated by the plus sign and alkaline media by the minus sign, and that the degree of acidity or alkalinity be noted in parts per hundred; thus a medium marked +1.5 would indicate that the medium was acid and that 1.5 % of n/1 sodium hydroxid is required to make it neutral to phenolphthalein, while -1.5 would indicate that the medium was alkaline and that 1.5 % of n/1 acid must be added to make it neutral to the indicator.

Limits of accuracy of the proposed method for the control of the reaction of media :

The available data are as yet insufficient to warrant any conclusions upon this point. The limits of accuracy seem to vary with the ingredients employed in preparing nutrient media, different samples of meat infusion, pepton, and gelatin appearing to react differently with the acids and alkalis and in a way which is not understood.

This method, nevertheless, when carefully carried out, and when the media before titration are thoroughly mixed and are of the prescribed volume, give fairly uniform results.

Standard reaction of media (provisional) :

Experience seems to vary somewhat as to the optimum degree of reaction which shall be uniformly adopted in the preparation of standard culture media. To what extent this is due to variation in natural conditions as compared with variations of laboratory procedure, it seems impossible to state. Somewhat different degrees of reaction for optimum growth are required, not only in or upon the media of different composition and by bacteria of different species, but also by bacteria of the same species when in different stages of vitality.

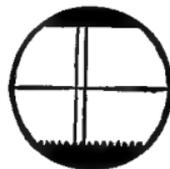
The bulk of available evidence from both Europe and America points to a reaction of +1.5 as the optimum degree of reaction for bacterial development in inoculated culture media; and while this experience is at variance with that in several of our own laboratories, it has been deemed wise to adopt +1.5 as the provisional standard reaction of media, but with the recommendation that the optimum growth reaction be always recorded in species descriptions."

II

THE OCULAR MICROMETER AND MICROMETRY¹

“**Ocular Micrometer, Eye-piece Micrometer.**— This, as the name implies, is a micrometer to be used with the ocular. It is a micrometer on glass, and the lines are sufficiently coarse to be clearly seen by the ocular. The lines should be equidistant and about $\frac{1}{10}$ or $\frac{1}{20}$ mm. apart, and every fifth line should be longer and heavier to facilitate counting. If the micrometer is ruled in squares (net micrometer) it will be very convenient for many purposes.

The ocular micrometer is placed in the ocular, no matter what the form of the ocular (*i.e.* whether positive or negative), at the level at which the real image is formed by the objective, and the image appears to be immediately upon or under the ocular micrometer, and hence the number of spaces on the ocular micrometer required to measure the real image may be read off directly. This is measuring the size of the real image, however, and the actual size of the object can only be determined by determining the ratio between the size of the real image and the object. In other words, it is necessary to get the valuation of the ocular micrometer in terms of a stage micrometer.



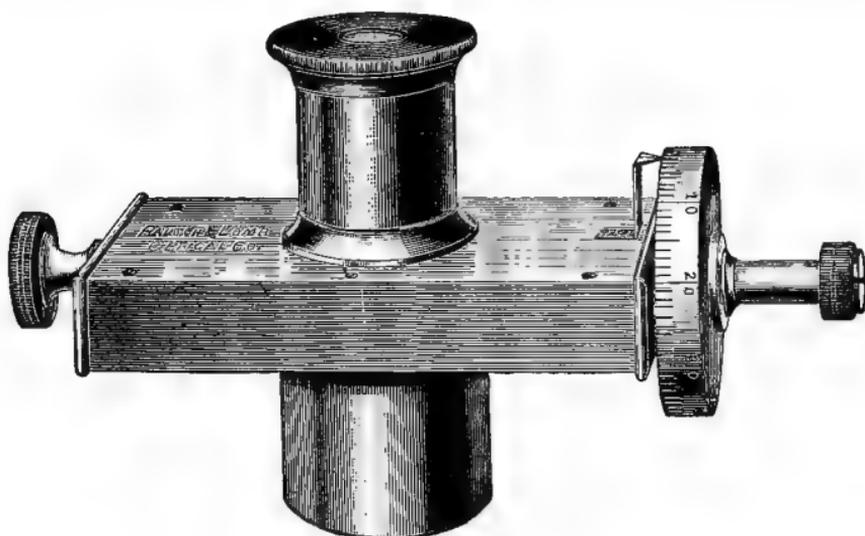
Field of large filar micrometer showing cross hairs and recording comb.

Valuation of the Ocular Micrometer.— This is the value of the divisions of the ocular micrometer for the purpose of micrometry, and is entirely relative, depending upon the magnification of the real image formed by the objective; consequently it changes with every change in the magnification of the real image, and must be specially determined for every optical combination (*i.e.* objective and ocular) and for every change in the length of the tube of the microscope, that is, it is necessary to determine the ocular micrometer valuation for every condition modifying the real image of the microscope (152).

¹These paragraphs are from Professor S. H. Gage's work on the microscope, published here by his consent. The references to sections are to the seventh edition of *The Microscope*.

Any Huygenian ocular may, however, be used as a micrometer ocular by placing the ocular micrometer at the level of the ocular diaphragm, where the real image is formed. If there is a slit in the side of the ocular, and the ocular micrometer is mounted in some way, it may be introduced through the opening in the side. When no side opening exists, the mounting of the eye-lens may be unscrewed and the ocular micrometer, if on a cover-glass, can be laid on the upper side of the ocular diaphragm.

Obtaining the Valuation of the Filar Micrometer. — This micrometer (Figs. 98–99) consists of a Ramsden's ocular and cross lines.



Filar Micrometer.

As seen in Fig. 98 there are three lines. The horizontal and one vertical line are fixed. One vertical line may be moved by the screw back and forth across the field.

For obtaining the valuation of this ocular micrometer an accurate stage micrometer must be used. Carefully focus the $\frac{1}{100}$ mm. spaces. The lines of the ocular micrometer should also be sharp. If they are not, focus them by moving the top of the ocular up or down (164). Make the vertical lines of the filar micrometer parallel with the lines of the stage micrometer. Take the precautions regarding the width of the stage micrometer lines given in 167.

Note the position of the graduated wheel and of the teeth of the recording comb, and then rotate the wheel until the movable lines traverse one space on the stage micrometer. Each tooth of the recording comb indicates a total revolution of the wheel, and by noting the number of teeth required and the graduations on the wheel, the revolutions and parts of revolution required to measure the $\frac{1}{100}$ mm. of the stage micrometer can be easily noted. Measure in like manner four or five spaces and get the average. Suppose this average is $1\frac{1}{4}$ revolutions, or 125 graduations, on the wheel, to measure the $\frac{1}{100}$ mm., or 10μ (157), then one of the graduations on the wheel would measure 10μ divided by $125 = .08\mu$. In using this valuation for actual measurement, the tube of the microscope and the objective must be exactly as when obtaining the valuation (165).

Example of Measurement. — Supposing one used the red blood corpuscles of a dog, or monkey, etc., every condition being as when the valuation was determined, one notes very accurately how many of the graduations on the wheel are required to make the movable lines traverse the object from edge to edge. Suppose it requires 94 of the graduations to measure the diameter, the actual size of the corpuscle would be $94 \times .08\mu = 7.52\mu$.

The advantage of the filar micrometer is that the valuation of one graduation being so small, even the smallest object to be measured would require several graduations to measure it. In ocular micrometers with fixed lines small objects like bacteria might not fill even one space; therefore estimations, not measurements, must be made. For large objects, like most of the tissue elements, the ocular micrometers with fixed lines answer very well, for the part which must be estimated is relatively small, and the chance of error is correspondingly small.

Obtaining the Ocular Micrometer Valuation for an Ocular Micrometer with Fixed Lines (Figs. 33, 34, p. 25). — Use the stage micrometer as object. Light the field well and look into the microscope. The lines of the ocular micrometer should be very sharply defined. If they are not, raise or lower the eye-lens to make them so, that is, focus as with the simple magnifier.

When the lines of the ocular micrometer are distinct, focus the microscope (45, 46, 56) for the stage micrometer. The image of the stage micrometer will appear to be directly under or upon the ocular micrometer.

Make the lines of the two micrometers parallel by rotating the ocular or changing the position of the stage micrometer, or both if necessary, and then make any two lines of the stage micrometer coincide with any two on the ocular micrometer. To do this it may be necessary to pull out the draw tube a greater or less distance. See how many spaces are included on each of the micrometers.

Divide the value of the included space or spaces on the stage micrometer by the number of divisions on the ocular micrometer required to include them, and the quotient so obtained will give the valuation of the ocular micrometer in fractions of the unit of measure of the stage micrometer. For example, suppose the millimetre is taken as the unit for the stage micrometer, and this unit is divided into spaces of $\frac{1}{10}$ and $\frac{1}{100}$ mm. If now, with a given optical combination and tube length, it requires 10 spaces on the ocular micrometer to include the real image of $\frac{1}{10}$ mm. on the stage micrometer, obviously 1 space on the ocular micrometer would include only one-tenth as much, or $\frac{1}{10}$ mm. $10 = \frac{1}{\frac{1}{10}}$ mm., that is, each space on the ocular micrometer would include $\frac{1}{100}$ of a millimetre on the stage micrometer, or $\frac{1}{10}$ mm. of length of any object under the microscope, the conditions remaining the same. Or, in other words, it would require 100 spaces on the ocular micrometer to include 1 mm. on the stage micrometer, then as before 1 space of the ocular micrometer would have a valuation of $\frac{1}{100}$ mm. for the purposes of micrometry; and the size of any minute object may be determined by multiplying this valuation of 1 space by the number of spaces required to include it. For example, suppose the fly's wing or some part of it covers 8 spaces on the ocular micrometer, it would be known that the real size of the part measured is $\frac{1}{100}$ mm. $\times 8 = \frac{8}{100}$ mm. or 80μ (157).

Varying the Ocular Micrometer Valuation. — Any change in the objective, the ocular, or the tube length of the microscope, that is to say, any change in the size of the real image, produces a corresponding change in the ocular micrometer valuation (152, 161)."

III

ANIMAL INOCULATION FOR PURPOSES OF DIAGNOSIS

It is not always possible by the ordinary culture methods to successfully determine the specific nature of a disease from a small piece of affected organ or tissue of the diseased animal or man. In making a positive diagnosis, therefore, it is often necessary to resort to animal inoculation. This is done by injecting into the animal chosen a small quantity of the tissue or fluid supposed to contain the virus of the specific disease, such as tuberculosis, glanders, rabies, and often of swine plague, hog cholera, anthrax, diphtheria, and others. Animal inoculation is further demandatory in determining the degree of virulence of pathogenic bacteria, the strength of toxins, antitoxins, etc. In other words, the living animal must for the present serve in certain instances as a testing reagent. The fact should be kept in mind that the lesions produced in the experimental animal are not necessarily and in most cases they are not the same as those in the animal (or man) from which the virus was obtained. It is the rule, however, that each virus produces characteristic lesions from which the disease can usually be diagnosed in the smaller animal.

Animals Used. — For simple diagnostic work the guinea pig and rabbit are usually employed, although white and gray mice, dogs, and other animals are sometimes used.

Method. — In preparing the animal for inoculation the hair should be removed over the area of operation by the use of scissors, and the skin washed and disinfected. A solution of corrosive sublimate, 1 to 1000, or a 5% solution of carbolic acid, may be used. The incision should be made with a sharp knife. Liquid material is usually injected with a hypodermic syringe. An anæsthetic should be given whenever the pain inflicted is to be long continued or excessively severe. The place of inoculation should be chosen where a local swelling, infiltration of tissue, or abscess would not interfere with the animal's locomotion.

SPECIFIC DISEASES FOR WHICH ANIMAL INOCULATIONS ARE MOST COMMONLY RESORTED TO FOR DIAGNOSTIC PURPOSES

Tuberculosis. — Guinea pigs are preferable, although rabbits may be used. With tuberculous tissues either of two methods may be employed. (1) A small piece (about the size of a pea or bean) of the tissue may be inserted under the skin by first making an incision with a sharp scalpel through the skin and superficial fascia and then with a pair of fine forceps insert the bit of tissue well under the skin and close the opening with one or more sutures. (2) The tissue may be crushed in a mortar and thoroughly mixed with a few cubic centimetres of sterile water or bouillon and then injected with a hypodermic syringe. The needle should be of large calibre. If it is suspected milk, it may be injected into the abdominal cavity. If the material is tuberculous and contains living tubercle bacteria, the death of the animal follows in from three weeks to four months. Usually the lymphatic glands of the groin and axilla are enlarged and often caseous. If a guinea pig is used, the liver, spleen, lungs, and kidneys are liable, in the order named, to be affected; if a rabbit, the lungs are often the first of the visceral organs to be attacked. (See pathology for description of tissue changes.)

Glanders. — Male guinea pigs should be used. The material usually consists of the nasal discharge from the suspected glandered horse, or bits of scrapings from the ulcers, or pieces of affected tissue. The method to be followed is precisely the same as with the subcutaneous injection of tuberculous material. In these cases there is liable to be a local swelling and abscess. The first indication of glanders noticed is usually orchitis. The lymphatic glands in the groin are also enlarged. After the orchitis becomes well marked the guinea pig may be chloroformed and examined. Pure cultures of the specific organism can be obtained in most cases from the suppurating focus in the testicle. The spleen is usually enlarged and sprinkled with grayish nodules. Other organs may be involved.

Rabies. — The method usually followed in diagnosing rabies is to inoculate a rabbit, guinea pig, or dog beneath the dura with a bit of the brain or spinal cord of the suspected rabid animal. Other methods are being introduced and the guinea pig is reported by some to respond more promptly, but in my experience the subdural

inoculation of rabbits has been most reliable. The injection through the optic foramen has been tried. The subdural method is, briefly stated, as follows :

The brain of the suspected animal is removed with aseptic precautions as soon as possible after death. A small piece of the brain or spinal cord is placed in a sterile mortar and thoroughly ground with a few cubic centimetres of sterilized water or bouillon. This forms the suspension to be injected. The hands of the operator and all instruments are carefully disinfected. The rabbit is etherized, the hair clipped from the head between the eyes and ears, and the skin thoroughly washed and disinfected. A longitudinal incision is then made, the skin and subcutaneous tissue held back by means of a tenaculum, a crucial incision is made in the periosteum on one side of the median line to avoid hemorrhage from the longitudinal sinus, and the four corners of the periosteum reflected or pushed back. By the aid of a trephine a small button of bone is easily removed, leaving the *dura mater* exposed. With a hypodermic syringe a drop or more of the rabid brain suspension is injected beneath the *dura*, the periosteum is replaced, the skin carefully sutured and disinfected, and the rabbit returned to its cage. As soon as the influence of the anæsthetic has passed off, the rabbit shows no appearance of discomfort. If the operation is performed in the forenoon, the animal partakes of its evening meal with the usual relish. The inoculation wound heals rapidly and the rabbit exhibits *every appearance of being in perfect health* until the beginning of the specific symptoms, which occur ordinarily in from 15 to 30 days, usually in about 20 days after the inoculation. Occasionally the symptoms appear earlier than 15 days and in some cases the rabbits are not attacked for from 1 to 3 months.

The symptoms following the inoculations have in my experience been quite uniform, the only pronounced difference being in the length of time the rabbits lived after the initial manifestation of the disease. The fact should be clearly stated that rabbits do not ordinarily become furious. In some instances they are somewhat nervous for a day or two preceding the paralysis. There appears to be a marked hyperæsthesia. Usually the first indication of the disease is a partial paralysis of one or both hind limbs. This gradually advances until the rabbits are completely prostrated, the only evidence of life being a slight respiratory move-

ment. The head occupies different positions. In some it is drawn back as in tetanus; in others it is drawn down with the nose near the fore legs; and in still others it is extended as if the animal were sleeping. The period of this complete paralysis varies from a few hours to a few days, but ordinarily it does not exceed 24 hours. Although the animals are unable to move voluntarily, there is usually a reflex action of the limbs until a very short time before death.

During the period of incubation the temperature of the rabbits remains normal. As the time approaches for the first symptoms to appear there has been in the animals tested an elevation of temperature of from 1 to 2 degrees, which continued for a variable length of time, but rarely longer than 2 days. This is followed by a gradual or usually a more rapid drop to the subnormal, which continues to the end.

Swine Plague.—Rabbits are most susceptible. Inoculate subcutaneously with a bit of the pneumonic tissue, either in a solid piece or in a suspension in bouillon hypodermically. In case of virulent swine-plague bacteria, the rabbit will die in from 16 to 36 hours from septicæmia. Pure cultures can be obtained from the blood, spleen, liver, or kidney. Stained cover-glass preparations from these organs show a greater or less number of polar-stained bacteria.

In case of a more attenuated virus the rabbit will live from a few days to several weeks and possibly months. In these cases there are usually marked local cell infiltrations, with inflammation of one or more of the serous membranes and possibly metastatic abscesses.

Hog Cholera.—Rabbits are most desirable. They are inoculated in the same manner as with tuberculosis. With ordinarily virulent bacteria the rabbits will die in from seven to ten days. The lesions are essentially a purulent infiltration of the subcutis at the point of inoculation, an enlarged and very dark colored spleen, and areas of coagulation necrosis in the liver. Pure cultures can be obtained from the blood, liver, or spleen. *1/2 cc. 18 Hour culture.*

Anthrax.—Mice or guinea pigs should be used. They are inoculated in the manner described above. They die of septicæmia usually in from 24 to 72 hours. It is not commonly necessary to resort to animal inoculation with this disease. Occasionally, however, it is a very necessary procedure in making an early diagnosis.

Diphtheria.—Guinea pigs are nearly always used. In certain rare

cases of mixed cultures taken directly from the suspected throat it is desirable to inoculate one or more guinea pigs to determine whether the suspected organism present is a virulent Klebs-Loeffler bacterium. In these cases a suspension of the growth on the serum may be injected. The guinea pig dies usually in from 36 to 80 hours. The lesions produced have been described by Park as follows :

“At the seat of inoculation there is a grayish focus surrounded by an area of congestion ; the subcutaneous tissues for some distance around are œdematous ; the adjacent lymph nodes are swollen ; and the serous cavities, especially the pleural and the pericardial, frequently contain an excess of fluid, usually clear, but at times turbid ; the lungs are generally congested. In the organs are found numerous smaller and larger masses of necrotic cells, which are permeated by leucocytes. The heart and voluntary muscle fibres usually show degenerative changes. Occasionally there is fatty degeneration of the liver and kidneys. The number of leucocytes in the blood is increased. From the area surrounding the point of inoculation, virulent bacilli may be obtained, but in the internal organs they are only occasionally found, unless an enormous number of bacilli have been injected. Paralysis, commencing usually in the posterior extremities, and then gradually extending to other portions of the body and causing death by paralysis of the heart or respiratory organs, is also produced in many cases in which the inoculated animals do not succumb to a too rapid intoxication.”

Guinea pigs are used for testing the virulence of pure cultures and the strength of the toxin and antitoxin. For further details, see text-books of bacteriology.

IV

CULTIVATION OF BACTERIUM (BACILLUS) TUBERCULOSIS

The isolation of this organism from tuberculous lesions and getting it to multiply readily on artificial media necessitates a very special and careful procedure. When it becomes accustomed to artificial media its continued cultivation is not difficult. Dr. Theobald Smith, of Harvard University (*Jour. of Exp. Med.*, Vol. III., 1898, p. 451), has the credit of formulating a method by combining details in such a manner that the procuring of cultures is, in most cases, possible. Dog serum is used. The method, as he gives it, is as follows, viz. :

“The dog was bled under chloroform and the blood drawn from a femoral artery, under aseptic conditions, through sterile tubes directly into sterile flasks. The serum was drawn from the clots with sterile pipettes and either distributed at once into tubes or else stored with 0.25 to 0.3% chloroform added. Discontinued sterilization was rendered unnecessary. The temperature required to produce a sufficiently firm and yet not too hard and dry serum is for the dog 75° to 76° C. For horse serum it is from 4° to 5° lower. The serum was set in a thermostat into which a large dish of water was always placed to forestall any abstraction of moisture from the serum. About 3 hours suffice for the coagulation. When serum containing chloroform is to be coagulated, I am in the habit of placing the tubes for an hour or longer in a water bath at 55° to 60° C., or under the receiver of an air pump, to drive off the antiseptic. This procedure dispenses with all sterilization excepting that going on during the coagulation of the serum. It prevents the gradual formation of membranes of salts, which, remaining on the surface during coagulation, form a film unsuited for bacteria. Tubes of coagulated serum should be kept in a cold closed space where the opportunities for evaporation are slight. They should always be kept inclined.

The ordinary cotton-plugged test tubes I do not use, because of the rapid drying out permitted by them, as well as the opportunities for infection with fungi. Instead, a tube is used which has a ground glass cap fitted over it. This cap contracts into a narrow tube

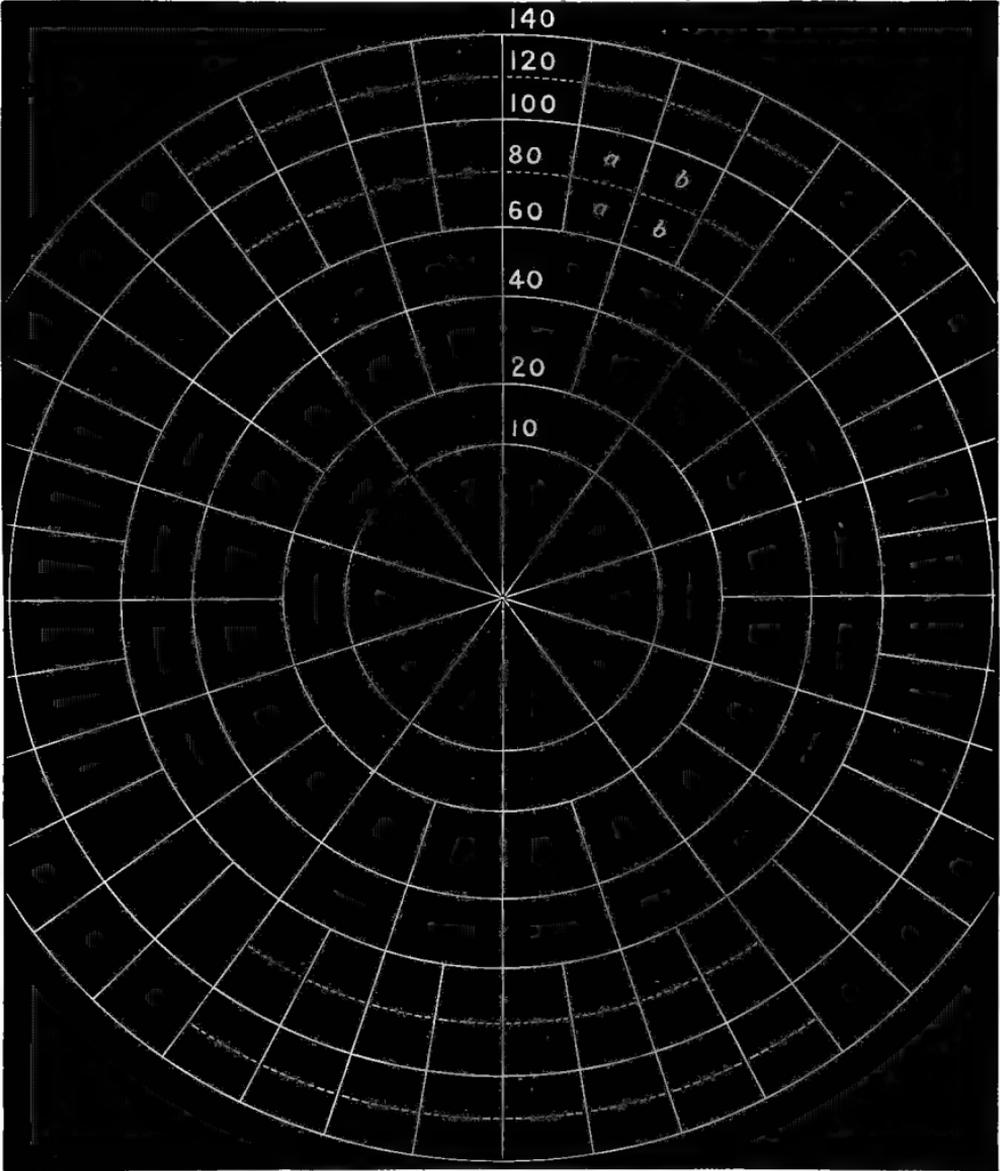
plugged with glass wool. This plug is not disturbed. The tube is cleaned, filled, and inoculated by removing the cap. With sufficient opportunity for the interchange of air little evaporation takes place, and contamination of the culture is of very rare occurrence. In inoculating these tubes, bits of tissue, which include tuberculous foci, especially the most recent, are torn from the organs and transferred to the serum. Very little crushing, if any, is desirable or necessary. I think many failures are due to the often futile attempts to break up firm tubercles. Nor should the bits of tissue be rubbed into the surface, as is sometimes recommended. After a stay of several weeks in the thermostat, I usually remove the tubes and stir about the bits of tissue. This frequently is the occasion for a prompt appearance of growth within a week, as it seems to put certain still microscopic colonies in or around the tissue into better condition for further development. The thermostat should be fairly constant, as urged by Koch in his classic monograph, but I look upon moisture as more important. If possible, a thermostat should be used which is opened only occasionally. Into this a large dish of water is placed, which keeps the space saturated. Ventilation should be restricted to a minimum. As a consequence, moulds grow luxuriantly and even the gummed labels must be replaced by pieces of stiff manila paper fastened to the tube with a rubber band. By keeping the tubes inclined, no undue amount of condensation water can collect in the bottom, and the upper portion of the serum remains moist. The only precaution to be applied to prevent infection with moulds is to thoroughly flame the joint between tube and cap as well as the plugged end, before opening the tube. When test tubes are employed it is well to dip the lower end of the plug into sterile molten paraffin and to cover the tube with a sterilized paper cap. The white bottle caps of the druggist are very serviceable."

Unless the tuberculous material is perfectly fresh (uncontaminated), and in the early stages of the disease, it is safer to inoculate a guinea pig, and after the lesions begin to develop to chloroform it and make the cultures from the recently affected liver or spleen.

JEFFERS' PLATE

FOR COUNTING COLONIES OF BACTERIA IN PETRI DISHES.

The area of each division is one square centimeter.



V

THE METRIC SYSTEM.



10 CENTIMETER RULE.

The upper edge is in millimeters, the lower in centimeter and half centimeters.

UNITS. *The most commonly used divisions and multiples.*

THE METER FOR LENGTH . . .	{	Centimeter (cm.), 1/100th meter; millimeter (mm.), 1/1000th meter; micron (μ), 1/1000th millimeter; the micron is the unit in Micrometry. Kilometer, 1000 meters; used in measuring roads and other long distances.
THE GRAM FOR WEIGHT . . .	{	Milligram (mg.), 1/1000th gram. Kilogram, 1000 grams; used for ordinary masses.
THE LITER FOR CAPACITY . . .	{	Cubic centimeter (cc.), 1/1000th liter. This is more common than the correct form, milliliter.

Divisions of the Units are indicated by the Latin prefixes: *deci*, 1/10th; *centi*, 1/100th; *milli*, 1/1000th.

Multiples are designated by Greek prefixes: *deka*, 10 times; *hecto*, 100 times; *kilo*, 1000 times; *myria*, 10,000 times.

Table of Metric and English Measures.

Meters = 100 centimeters, 1000 millimeters, 1,000,000 μ , 39.3704 inches.
 Millimeter (mm.) = 1000 microns, 1/10th millimeter, 1/1000th meter, 1/25th inch, approximately.

Micron (μ) (Unit of Measure in Micrometry) = 1/1000th mm., 1/1000000th meter (0.000039 inch), 1/2500th inch, approximately.

Inch (in.) = 25.399772 mm. (25.4 mm., approx.).

Liter = 1000 milliliters or 1000 cubic centimeters, 1 quart (approx.).

Cubic centimeter (cc. or cctm.) = 1/1000th of a liter.

Fluid ounce (8 fluidrachms) = 29.578 cc. (30 cc., approx.).

Gram = 15.432 grains.

Kilogram (kilo) = 2.204 avoirdupois pounds (2 1/5th pounds, approx.).

Ounce avoirdupois = (437 1/2 grains) = 28.349 grams.

Ounce troy or apothecary's = (480 grains) = 31.103 grams.

Temperature.

To change Centigrade to Fahrenheit: $(C. \times 9/5) + 32 = F.$ For example, to find the equivalent of 10° Centigrade, $C. = 10^\circ$
 $(10^\circ \times 9/5) + 32 = 50^\circ F.$

To change Fahrenheit to Centigrade: $(F. - 32^\circ) \times 5/9 = C.$ For example, to reduce 50° Fahrenheit to Centigrade, $F. = 50^\circ$, and $(50^\circ - 32^\circ) \times 5/9 = 10^\circ C.$; or -40 Fahrenheit to Centigrade, $F. = -40^\circ$ ($-40^\circ - 32^\circ$) = -72° , whence $-72^\circ \times 5/9 = -40^\circ C.$

(From "The Microscope," by Prof. S. H. Gage, used here with his permission.)

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EXERCISE XXVIII.

A STUDY OF BACTERIA IN MILK.

It is desirable to understand somewhat clearly the bacterial contents of milk and to know something of the physiological properties of these bacteria. For this reason it is desirable to study though but briefly the bacteria in ordinary market milk.

References.—Dairy Bacteriology, Russell; also one by Conn, and one by Grotenfelt. Hunziker, Germicidal action in cows milk. Bulletin No. 197, Cornell Univ. Exp. Station. Ward, The invasion of the udder by bacteria. Bulletin 178, *Ibid.* Park, Bacterial contamination of the milk of our cities. The N. Y. Univ. Bulletin of the Med. Science, Vol. 1. Moore, Bacteria in milk. Report of the Com. of Agriculture of N. Y., 1902.

Work for this Exercise.—Examine the plate cultures made from milk. Describe the different kinds of colonies and state approximately the number of each. Examine microscopically the bacteria in one of each kind of colonies and determine its genus.

Inoculate a tube of milk and one of gelatin from each of three different kinds of colonies, stating the genus of the bacteria in each. *3 milk, 3 gelatin*

Describe the cultures of *B subtilis*. *last.*

Inoculate groups A and B of media from a culture of *B. prodigiosus* furnished. *found in air, soil water*

Make for examination at the next exercises ~~a series of~~ three gelatin plates from a sample of water furnished; (unfiltered creek or well water) using the quantity for each culture designated by the instructor.

