BACTERIOLOGY AND DIAGNOSIS

BY

MOORE AND FITCH

EXERCISES IN BACTERIOLOGY AND DIAGNOSIS

FOR VETERINARY STUDENTS AND PRACTITIONERS

BY

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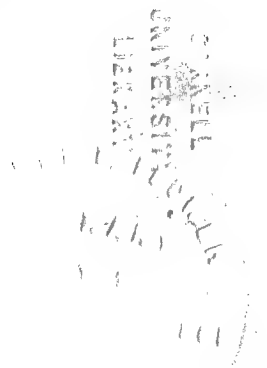

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PREFATORY NOTE

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 and protozoölogy 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 exercises 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 microbiology in the practice of veterinary medicine. They aim to impart a definite knowledge of a few important species of microörganisms and a working knowledge of certain of the more essential methods. Following the regular laboratory exercises are directions for the diagnosis of several specific diseases. These are based largely on the instruction given in the previous exercises.

THE AUTHORS

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ABBOTT, A. C. The Principles of Bacteriology.

ABEL, R. Taschenbuch für den bakteriologischen usw.

ARCHINARD and PEDERSEN. Microscopy and Bacteriology.

BAUMGARTEN. Jahresbericht ü. d. Fortsch. d. path. Mikroorganismen.

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CROOKSHANK, E. M. Text-Book of Bacteriology and Infectious Diseases.

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HEKTER, C. A. The Common Bacterial Infections of the Digestive Tract.

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MATZUSCHITA, T. Bacteriologische Diagnostik.
MOORE, V. A. The Principles of Microbiology.
MOSSELMAN and LIÉNAUX. Manual of Veterinary Microbiology.
MCFARLAND, J. A Text-Book upon the Pathogenic Bacteria.
MIGULA, W. System der Bakterien.
MUIR and RITCHIE. Manual of Bacteriology.
NEWMAN, G. Bacteria.
NOVY, F. G. Laboratory Work in Bacteriology.
PARK, W. H., and WILLIAMS, A. W. Pathogenic Bacteria and Protozoa.
PEARMAIN and MOOR. Applied Bacteriology.
SMITH, E. F. Bacteria in Relation to Plant Diseases. 2 vols.
STERNBERG, GEORGE M. Text-Book of Bacteriology.
STERNBERG, GEORGE M. A Manual of Bacteriology.
SWITHINBANK and NEWMAN. Bacteriology of Milk.
THOINOT and MASSELIN. Précis de microbie.
WILLIAMS, H. U. A Manual of Bacteriology.
WOODHEAD, G. S. Bacteria and their Products.
WURTZ, R. Précis de bactériologie clinique.

JOURNALS AND PERIODICALS OF SPECIAL VALUE TO THE STUDENT OF BACTERIOLOGY

American Journal of Public Health.
Annales de l'institut Pasteur.
Annales de médecine vétérinaire.
Archiv für wissenschaftliche und praktische Tierheilkunde.
Archives de médecine expérimentale et d'anatomie pathologique.
Centralblatt für Bakteriologie, Parasitenkunde u. Infektionskrankheiten.
Jahresbericht über die Fortschritte in der Lehre von den pathogenen Mikroorganismen.
Monatshefte für praktische Tierheilkunde.
Journal of Comparative Pathology and Therapeutics.
The Journal of Experimental Medicine.
The Journal of Infectious Diseases.
The Journal of Medical Research.
The Journal of Pathology and Bacteriology.
Journal of Royal Microscopical Society, London.
Journal of Tropical Medicine and Hygiene.
Zeitschrift für Hygiene u. Infectionskrankheiten.
Zeitschrift für Immunitätsforschung und experimentelle Therapie.
Zeitschrift für Infektionskrankheiten parasitäre Krankheiten und Hygiene der Haustiere.

BOOKS VALUABLE FOR METHODS AND FORMULÆ

Ehrlich and others. Enzyklopädie der mikroskopischen Technik.
Gage. The Microscope.
Lee. The Microtomist's Vade-Mecum.
Lafar. Technical Mycology.
Mallory and Wright. Pathological Technique.
Saccardo. Chromotaxia seu Nomenclator Colorum. Patavii, 1894.
Von Kahlden. Methods of Pathological Histology.

APPARATUS AND MATERIAL

Apparatus. The apparatus and material for use in bacteriology in a student laboratory fall very naturally into three groups, viz.: (1) apparatus to be used in common by all students, and for which no individual is responsible excepting when in actual use by him, and the supplies from which each student draws the necessary quantity for the work assigned; (2) apparatus to be assigned to each student for personal use and for which he is wholly responsible; and (3) material such as notebooks to be provided by each. The assignment of equipment and supplies in accordance with this plan has been made in a general way, as indicated in the following paragraphs.

(1) **Apparatus in laboratory for general use.** This includes the apparatus and chemicals to be used in common by all students, and 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 aid in counting colonies, micrometers, metric rules, burettes, tripods, funnels, beakers, pipettes, graduates, glass tubing and rods, also the chemicals necessary for carrying on the work, such as various acids and alkalies, disinfectants, alcohol, aniline dyes, and 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 being furnished as needed. The equipment also includes color charts and the more important books of reference.

(2) **Apparatus furnished for individual use.** The various appliances used by each student and for which he becomes personally responsible are a microscope with substage condenser, 2 oculars (1 and 2 inch) and 3 objectives ($\frac{2}{3}$, $\frac{1}{6}$, and $\frac{1}{12}$ inch), a bottle of immersion oil, a hand magnifier, 75 small test tubes, 30 large test tubes, 10 fermentation tubes, 18 Petri dishes, one 1000 cc. and two 500 cc. Erlenmeyer flasks, 7 one-ounce bottles for reagents and stains, supplied with pipettes or glass rods, 1 platinum-wire loop, 1 platinum-wire needle, 3 tin cups for holding cultures, 3 wire baskets 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 ointment jars 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.

(3) **Material to be provided by each student.** A box of slides and cover glasses (cover glasses 18 mm. square 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. W. Faber's blue pencil for marking on glass, fine forceps for handling cover glasses, and paper for laboratory notes with manila cardboard covers or suitable notebooks.

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, or that of an alcohol lamp, 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.

INTRODUCTION

Plan of exercises. Bacteriology has become one of the recognized branches in the curriculum of all medical and veterinary colleges. In many universities it is taught as a part of the course in general biology. It is, however, still a young science and the best methods for teaching it have not as yet been determined. All are agreed, however, that in addition to such text-book work and lectures as may be required there should be laboratory practice in actually handling and studying various bacteria and in determining their special morphological characters and physiological properties.

In order to differentiate the various species of bacteria, to isolate them from impure cultures or animal tissues, it is necessary that one should be familiar with the methods to be used; otherwise the attention will be directed more to the *modus operandi* than to the organisms themselves. On this point, however, there is much difference of opinion. Some laboratory workers believe that the methods should be taken up and learned in connection with the study of the various species of bacteria and thus avoid the loss of time that special instruction in methods requires. Others favor a devotion of a portion of the time to a consideration and drill in the methods to be employed later on in the course in the serious study of species and in diagnostic work.

The experience in this laboratory has been that the best results are obtained by teaching the more fundamental principles and methods as such before attempting to apply them in the study of the various species of bacteria or in practical diagnostic work. It has happened, even when the number of exercises is very limited, that a preliminary drill in the methods is greatly to the advantage of the student. From the nature of the subject, its application can be made and benefit derived therefrom only by those who know how to do the things that the exigencies of the moment demand. This means efficiency in knowing how. In following these directions, therefore, the student must understand that the purpose of the first twenty exercises is to teach him how to do the things called for in the later exercises in the study of species and in some of the practical applications of bacteriology.

Another feature of these directions is that they aim to teach the student how to study and observe bacteria in their cultures rather than to tell him what he is to observe. It is not intended that they should take the place of lectures and text-books in bacteriology. Their mission is to aid the student in finding out for himself what the text-books relate concerning certain species, and to guide him in the elementary steps in the more important diagnostic procedures, tests, and analyses.

The fact must also be recognized that in a short elementary course it is not possible to try several methods for doing the same thing. This restricts us to the use of a single procedure. The one is given that seems to us best adapted to the limited time and facilities of the student. It may happen, however, that other methods would be preferable under other

conditions or in the hands of certain individuals. This limitation is a necessity, however, in an elementary course of instruction. Its objection is partially met by references to text-books and other publications where additional methods are described. It is very important that the student familiarize himself with at least a few of the more important books and periodicals dealing with bacteriology and protozoa. They are the source to which he must go later for information on this subject, and consequently a knowledge of their nature and how to use them may be of unmeasured value.

The introduction of methods for the specific diagnosis of the more common infectious diseases of animals will, it is believed, be of much value to practitioners, especially after they have had the exercises as students.

EXERCISES IN BACTERIOLOGY AND DIAGNOSIS

FOR VETERINARY STUDENTS AND PRACTITIONERS

LABORATORY BACTERIOLOGY

EXERCISE I

CLEANING GLASSWARE

1. It is necessary that the glassware employed should be horoughly cleaned before it is used. Several special methods ıave been suggested for this purpose, but the one frequently :mployed by chemists seems to be the most easily handled ınd quite as efficient for general use as the more elaborate, pecialized processes. It consists in applying the chromic .cid cleaning mixture after washing the tubes and flasks with vater. Experience has taught that usually this method may ıe abandoned, as a strong alkali is quite sufficient to clean he ordinary glassware. It is sometimes necessary to employ nore special methods for cover glasses which are to be used n staining bacteria where a mordant is required. Only one ıf these special methods will be given here.

2. Work for this exercise. Clean all the glassware, test ubes, fermentation tubes, flasks, Petri dishes, and reagent ıottles assigned.

If the next laboratory period occurs on the following day, ılace the flasks and test tubes in the incubator to insure their ıeing dry before they are plugged.

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

Read the laboratory maxims.

3. Methods to be followed in cleaning the different appa-atus. (*a*) *Test tubes.* Wash the tubes carefully with a *strong* lkali soap and water, using the test-tube brush. Rinse the

tubes thoroughly in hot water and drain them, using a drainage board. [It is sometimes desirable to use the cleaning mixture.[1] In this case, after the tubes are washed with soap and water, they are stood in a glass jar (aquarium) and filled with the cleaning mixture. After it has acted for from 10 to 20 minutes, pour it back from the tubes into the bottle originally containing it. The tubes are then to be thoroughly rinsed and dried as before.]

(*b*) *Fermentation tubes.* Treat these with the cleaning mixture in the same manner as the test tubes. This is necessary because the brush cannot be used.

(*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 soapsuds, after which rinse them separately in tap water and drain. The cleaning mixture need not be used. After they are dry the two parts of the Petri dish should be put together.

(*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 the color disappears; then cover them with alcohol until needed, when they can be wiped with a soft linen cloth or with lens

[1] *Formula for chromic acid cleaning mixture.* Dissolve 80 grams of potassium dichromate ($K_2Cr_2O_7$) in 300 cc. of warm water; when all of the $K_2Cr_2O_7$ is dissolved and the solution cooled, add it slowly, with constant stirring, to 460 cc. 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.

paper. Sometimes there appears to be a film on the surface of the cover glasses which interferes in making hanging-drop preparations. Sometimes this can be overcome, after they are wiped out of the alcohol, by placing them in a Petri dish without the cover and heating them in the dry-air sterilizer at a temperature of 160° to 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 bouillon is spread upon a properly cleansed cover glass it does not roll up in droplets, but will remain in a thin, even layer on the surface.)

Slides can be cleaned very satisfactorily by washing them in hot soapsuds, rinsing them in hot 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 a 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 first highly recommended to me by Dr. Erwin F. Smith, and later modified by Johnston and Mack. First clean the cover glasses by the ordinary method, after which boil them in an agate cup or glass beaker in a 5% solution of caustic soda for 10 minutes. After cooling, rinse thoroughly in distilled water, place in a beaker, and cover with a 10% solution of hydrochloric acid

and boil for 10 minutes. Then pour off the acid and rinse the cover glasses, one at a time, using forceps for handling, in distilled water and finally in ether-alcohol. When needed, wipe with a piece of cheese cloth that has been properly prepared by soaking in a 5% solution of caustic soda for a few hours, then rinsed in water, and then placed for a few hours in dilute (10%) hydrochloric acid, after which it should be thoroughly rinsed in distilled water and dried. Before wiping the covers cleanse the hands thoroughly in soap and water, then in ether.

5. Laboratory notes. In this and all subsequent exercises careful notes should be taken on the work done. When, however, 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 methods for sterilizing apparatus and instruments in text-books.)

7. Work for this exercise. Plug all the tubes and flasks with absorbent cotton and sterilize them, together with the Petri dishes.

After they are sterilized, store them in the locker until they are needed. The Petri dishes must not be opened until they are used.

If the periods are short this exercise and the following one may be worked together.

8. Plugging the tubes and flasks. For this purpose absorbent cotton is used. Rolls of it 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 of equal firmness. (For method of closing the tubes more securely, see § **22.**)

9. Sterilizing glassware. (*a*) *Hot air.* Place all the test 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, not allowing it to rise 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.

(*b*) *Dry steam.* Sterilize the fermentation tubes in the autoclave at 15 lbs. pressure for half an hour. This method prevents a considerable amount of breakage, especially of the stand of the tube, that often occurs when these tubes are heated in the hot-air sterilizer.

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. It may be made directly from simple meat infusion or 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 cc. 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 routine work in the laboratory, bouillon prepared directly from the meat by macerating it for a short time at a high temperature (60° to 65° C.) is very satisfactory. The addition of peptone and the neutralization of the liquid are the same in both cases.

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

REFERENCES. Chapters on making culture media in text-books. Jour. of the Am. Public Health Asso., October, 1895, and January, 1898, p. 77. Smith, The Jour. of Exper. Med., Vol. III (1898), p. 647.

11. Work for this exercise. Make 1000 cc. of bouillon and distribute it as follows:

Put 5 cc. in each of 10 small sterile test tubes.

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

Put 5 cc. 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, add 1000 cc. (2 parts water to one of meat) of clear boiled water, cooled to 65° C., and stir thoroughly with a glass rod. Then macerate it, with frequent stirring, in a water bath at a temperature of 60° C. for one hour after the temperature of the meat and water reaches that of the water outside or, to save time, at a temperature of 65° C. for 30 minutes. Remove the meat by straining the liquid through a piece of cheese cloth. For this a stout iron meat press is desirable. Boil the strained meat infusion for 20 minutes.[1] Cool to harden the fat, and filter through filter paper. The filtrate should equal in quantity the amount of water used; if it does not, add enough distilled or clear boiled water to make up that amount. To this meat infusion add 1% peptone (Witte's) and ½% sodium chloride. Add enough of a normal solution of sodium hydrate to give the liquid a faintly alkaline reaction when litmus is used as an indicator. (For method of titrating media, see § **14.**) The infusion is then boiled in a water bath

[1] In case of a short period, the exercise may be divided at this point by boiling the infusion for 30 minutes and keeping it overnight. The receptacle should be covered.

r three quarters of an hour and filtered hot. The filtrate ıould be perfectly clear. The color will vary according to the nount of blood pigment in the meat used, and according to ıe length of time it is steamed or boiled, i.e. according to the nount of material precipitated out. After filtering, distribute ıe bouillon in tubes and flasks (see above), and stand them in wire basket for sterilization. Sterilize them by boiling in a osed water bath or steaming in the Arnold's steam sterilizer for ɔ minutes,[1] the time to be computed from the time the water ɔils or the temperature in the steamer reaches 99°. The ısks of bouillon should be boiled or steamed for 20 minutes ı each of the two succeeding days (certain anaërobic bac- ria may not be destroyed by this treatment). When they ıve cooled the outside of the tubes should be carefully wiped ith a moist cloth and placed in the incubator until the next boratory day. Then carefully examine them, and if any of ıe tubes are contaminated, that is, if the liquid is clouded or ıs a membrane on the surface, they must be rejected. Label ıe others and place them in the locker.

[1] The customary method of sterilizing culture media is to steam or ɔil them for about 10 minutes on each of 3 consecutive days. This ıs found very troublesome by the students, and, feeling that it was not ːcessary, a long series of test experiments was made by Mr. R. C. Reed, ıo found that 1 boiling or steaming for 30 minutes gave just as good sults as the customary 3 boilings. As the media are not used for 2 or days after sterilization, during which time they are kept in an incu- ıtor, the method is well suited to student laboratories, not for the ason that it saves time in preparing the media, but it relieves the ngestion in the sterilizer and appreciably aids the student. *When ːrilized by this method the media must not be inoculated for several ːys after their preparation or until they have stood in an incubator for least 18 hours to test their sterility.*

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

13. Labeling media and culture tubes. Stick on each tube of medium, 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.

14. Titration of bouillon. Take 5 cc. of bouillon and place in a porcelain evaporating dish with about 45 cc. of water. Boil for three minutes. Add 1 cc. of a solution of phenol-phthalein. Stir and add to the solution in the evaporating dish enough of a N/20 NaOH solution from a burette to give it a clear, bright pink color. This amount then of N/20 NaOH solution is required to neutralize 5 cc. of the bouillon. A provisional standard reaction of +1.5 to phenol-phthalein has been adopted. This would be slightly alkaline to litmus. In order to bring the entire amount of bouillon to the desired reaction (+ 1.5), subtract 1.5 from the amount of N/20 NaOH solution drawn from the burette and multiply the difference by the number of cc. of bouillon divided by 100. The product, if plus, represents the amount of N/1 NaOH to be added; if minus, the amount of N/1 HCL to be added. After mixing, test the reaction again in the same way and add alkali or acid if needed. For greater accuracy further tests should be made. (For a more complete discussion of the reaction of culture media, see Appendix I.)

EXERCISE IV

THE PREPARATION OF GELATIN AND AGAR

15. Of the solid media employed in cultivating bacteria, agar d gelatin are most commonly used. They depend for their ıtritive properties largely upon the bouillon from which they e made, the agar and gelatin forming simply the solidifying ements. The striking difference between the two is that the latin melts at the body temperature, whereas the agar is not adily liquefied below the boiling point. For this reason latin is not used as a solid medium for cultivating bacteria a high (body) temperature. There are several processes for eparing these media, but the addition of the dry agar and latin to bouillon (§ **12**) either immediately after it is filtered, later after it has been sterilized and stored in flasks, seems be the most convenient procedure. The agar itself is usu-y neutral in reaction, but the gelatin often has a decidedly id reaction. This necessitates the careful retesting of the action of the two media, even though neutral or slightly saline bouillon is used.

16. Work for this exercise. See that bouillon made in xercise III is properly sterilized. Prepare 300 cc. of gelatin d 300 cc. of agar, i.e. start with 300 cc. of bouillon for each. ıere will be considerable shrinkage owing to the amount lost the dishes, filter, etc., so that the quantities of media will be preciably less than this amount. Distribute each medium as llows:

Put 5 cc. in each of 10 small sterile test tubes.
Put 10 cc. in each of 12 large sterile test tubes.
Put the remainder in the flask which contained the bouillon.

17. The preparation of nutrient gelatin. Take a flask of uillon containing 300 cc. and pour it into a small agate-iron

dish, add 30 grams of sheet gelatin, and heat, 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 firmly 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[1] but ordinary filter paper which has been moistened with boiling water. 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 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 2 days. If the gelatin in any of the tubes becomes cloudy, the medium in those tubes must be rejected. Carefully wipe all the other tubes with a moist cloth, label, and place them in a locker, where they may be kept until used.

18. The preparation of nutrient agar. Weigh out 4.5 grams of agar and cut it into small pieces with a pair of scissors. Put the finely cut agar into an agate-iron dish, add 100 cc. of distilled water, and boil over a gas flame, with constant stirring to prevent scorching, until the agar is dissolved, giving a thick, homogeneous, pasty substance. Pour 300 cc. 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

[1] For illustrations and directions for folding filter paper, see Abbott's *Principles of Bacteriology*, 6th edition, p. 101. Filter paper already folded may be procured.

perature between 45° and 50° C., add the white of one , and thoroughly mix in the liquid agar. This is easily ac- plished by pouring it a number of times from one beaker another. When the egg albumen is dissolved the agar is rned to the water bath and boiled vigorously until the te of the egg is firmly coagulated. This usually takes about minutes. Filter the agar immediately, while hot, through yer of absorbent cotton which has been moistened with ing water. Distribute the filtrate in small and large tubes, directed. Sterilize, label, and store the agar in the same ner as the gelatin.

EXERCISE V

INOCULATING TUBES OF BOUILLON, AGAR, AND GELATIN

19. Work for this exercise. See that the media made in former exercises have been properly sterilized. 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 then to alcohol. Seal the agar slant and gelatin tubes.

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

20. 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 by pulling 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

gs replaced in their respective tubes, and the loop flamed put aside. Label the freshly inoculated tube with the ıe of the organism, source, and date. Stand it in a tray or and place it in the incubator.[1]

1. Inoculating tubes of agar. *Ordinarily the agar is ined before it is inoculated.* In this case it is spoken of as ined or slant agar. Occasionally the agar is inoculated with- inclining it. Cultures made in this manner are spoken of as ı or stick cultures. (*a*) *Inclined or slant agar.* Stand a tube gar in a wire basket in a water bath and boil it until the agar quefied. (To save repeating this it is well to incline the agar everal tubes which can be kept for future use, but after the ıts have been made for a long time it is better to boil and ant them, especially if they are to be used for organisms ch do not grow well on a dry surface.) Lay the tube on a ', the top resting on the side of the tray so that the surface of agar will be about 4 cm. long, and allow it to cool. In plac- the tubes the label should be up. When the agar has set, it ady for use. It is inoculated precisely as the bouillon; except t the loopful of culture is drawn over the inclined surface ead of being thrust into the medium as in the bouillon. Label . place it in the incubator with the inoculated bouillon tube. the following day there should be a grayish-white growth on surface of the agar covered by the loop. This is an agar ure of *B. coli.* (*b*) *Stab cultures.* These are made with a inum needle in the uninclined agar. The impregnated dle is pushed down through the center of the agar. In all er respects this culture is made like the slant-agar culture.

For illustrations and descriptions of different kinds of incubators, text-books. It is desirable to note especially the various burners thermoregulators employed to heat and regulate the temperature he incubators. Considerable information may also be acquired by fully looking through the catalogues of manufacturers and dealers acteriologic apparatus. Copies of some of these will be found on reference bookshelves.

22. Inoculating tubes of gelatin. Tube cultures in gelatin are usually made without inclining the gelatin, i.e. stab cultures. The tube of gelatin is inoculated in the same manner as the stab 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 along the needle track in about 2 days. This is a gelatin culture of *B. coli.*

23. 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 melt 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 fills the spaces between the fibers of cotton and also 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

24. 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 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.

25. 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 (viscidity).

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.

26. 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. (See Chester's terminology, §§ **31** and **51**.)

27. Testing the reaction of liquid cultures. Place a small piece of each of the red and blue litmus papers 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.

28. To determine the viscidity. (*a*) *Bouillon cultures.* Insert the platinum loop into the liquid and carefully withdraw it. The approximate degree of viscidity may be determined by the extent of the adhesion of the liquid to the loop and by the length of the threadlike 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. (*b*) *Agar and gelatin cultures.* Touch the surface growth with the end of the platinum needle, and if it is viscid, a threadlike string will be drawn out. Note whether the growth is pasty or friable.

29. Making hanging-drop preparations. (*a*) *From a bouillon culture.* Place a clean cover glass on a 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 on the use of the microscope, see *The Microscope*, by Professor S. H. Gage.) (*b*) *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 bouillon or sterilized water on the center. Touch the surface growth very gently with the end of the platinum 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).

30. 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 center 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 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?

31. Chester's terminology for descriptive bacteriology. Chester has introduced a terminology in descriptive bacteriology which has the advantage of being definite and concise, while at the same time it is sufficiently elastic to fit the varying

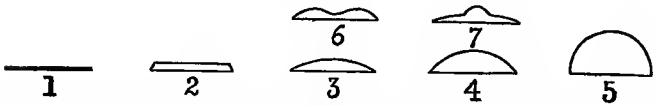


FIG. 1. Characters of surface elevation: 1, flat; 2, raised; 3, convex; 4, pulvinate; 5, capitate; 6, umbilicate; 7, umbonate.

forms of growth. It applies to the surface growth, to the growth along the needle track in the depth of the media, and to colonies on plate cultures.

1. Surface elevation. General character of surface growth as a whole.

Flat: thin, leafy, spreading over the surface.

Effused: spread over the surface as a thin, veilly layer, more delicate than the preceding.

Raised: growth thick, with abrupt terraced edges.

Convex: surface the segment of a circle, but very flatly convex.

Pulvinate: surface the segment of a circle, but decidedly convex.

Capitate: surface hemispherical.

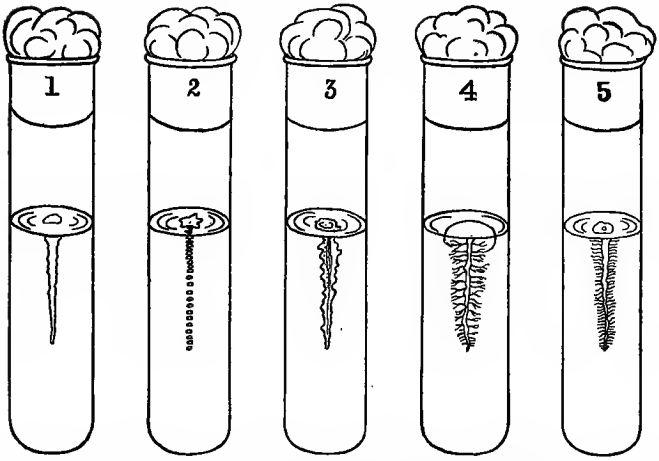


FIG. 2. Characters of growth in depth of media: 1, filiform; 2, beaded; 3, tuberculate-echinulate; 4, arborescent; 5, villous.

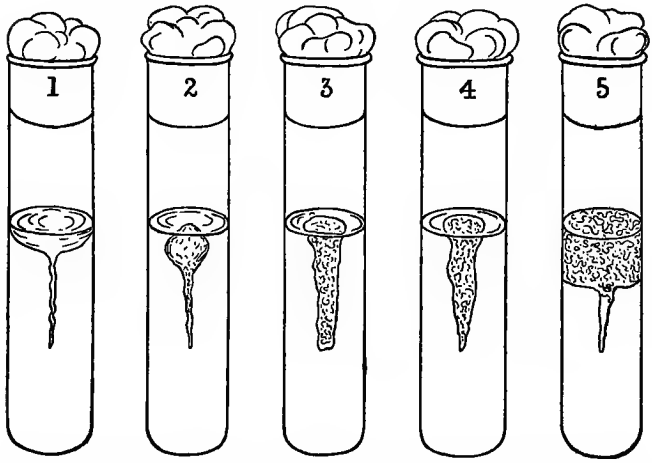


FIG. 3. Types of liquefaction in gelatin stab cultures: 1, crateriform; 2, napiform; 3, saccate; 4, infundibuliform; 5, stratiform.

2. Gelatin stab cultures. Nonliquefying line of puncture.

Filiform: uniform growth, without special character.
Nodose: consisting of closely aggregated colonies.
Beaded: consisting of loosely placed or disjointed colonies.
Papillate: beset with papillate extensions.
Echinate: beset with acicular extensions.
Villous: beset with short, undivided, hairlike extensions.
Plumose: a delicate feathery growth.
Arborescent: branched, or treelike, beset with branched hairlike extensions.

3. Gelatin stab culture. Liquefying line of puncture.

Crateriform: a saucer-shaped liquefaction of the gelatin.
Saccate: shape of an elongated sack, tubular, cylindrical.
Infundibuliform: shape of a funnel, conical.
Napiform: shape of a turnip.
Fusiform: outline of a parsnip, narrow at either end, broadest below the surface.
Stratiform: liquefaction extending to the walls of the tube and downward horizontally.

EXERCISE VII

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

32. 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 labeled to accompany notes.

Prepare the staining fluids used in this exercise from the formulæ given (§ **37**).

Read the paragraphs in the text-books on making and staining cover-glass preparations. See references and text-books for various staining solutions that are sometimes used.

33. Making cover-glass preparations. (*a*) *From bouillon cultures.* Place two 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 and, when dry, fix the bacteria to them 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 one second. After fixing they are ready for staining. (*b*) *From cultures on solid media* (*agar, gelatin, potato, serum, etc.*). Place the cover glasses on the tray, and on the center of each put a drop of sterile water or bouillon. Touch the surface growth of the culture with the end of the needle and then gently rinse it 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.

34. Staining bacteria in cover-glass preparations. (*a*) *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 it off 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 ($\frac{1}{6}$-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 and 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 a neutral or slightly alkaline Canada balsam.[1]

(*b*) *With carbol fuchsin.* Moisten the film side of the cover glass with water (using the pipette); then cover it with the stain and allow it to act for from 10 to 30 seconds. 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 for 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.

[1] To neutralize balsam, add some pure sodium carbonate to it and allow it to stand for about a month in a warm place, shaking it from time to time. Then allow the sodium to settle. The clear supernatant balsam will be found to be slightly alkaline.

Cover-glass preparations of bacteria are permanently mounted the same manner as similar preparations made from the ood or other tissues in histology, the process being to put a op of balsam on the center of the slide and place the prep- ation, film downward, over it and apply slight pressure. abel the preparation, giving the name of the organism, its urce (kind of culture, tissue, etc., from which the preparation as made), stain used, and date. If the specimen is not pre- rved, the slide and cover glass should be cleaned for future use.

35. Suggestions concerning the microscopic examination of ained preparations of bacteria. In the examination of the acteria in the stained condition the following points, and erhaps others, should be observed and noted. (*a*) *Concern- g their morphology.* Are they spherical, rod-shaped, or iral? Are they separated or united in clumps or chains? If d-shaped, are the ends pointed, round, or square? Are the acteria all of the same form and size? Note the presence absence of spores, granules, and capsules. (*b*) *Concerning eir reaction to staining fluids.* Do they stain uniformly or regularly? Do they stain deeply or faintly? Is the center ghter than the periphery? Are there an unstained central nd and deeply stained ends (polar stain)? Do all of the acteria take the stain alike?

36. Staining solutions. The basic aniline dyes are used in aining bacteria. There is a large number of these, and there e several formulæ for preparing staining solutions from ach. Further, as will be seen from the chapters on staining acteria in the text-books, there are several methods of apply- g these stains. In an introductory course, however, it is apossible to try them all, and consequently only those are escribed which seem to be the best adapted for general use. In addition to the ordinary staining solutions and methods ere are special processes for certain species, such, for exam- e, as the tubercle bacterium, and still others for staining rtain 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.

37. 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)

Saturated alcoholic solution of methylene blue	6 cc.
Caustic potash (1 % solution)	0.2 cc.
Distilled water	20 cc.

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 cc.
Carbolic acid (5 % solution)	100 cc.

Dissolve the fuchsin in the alcohol, after which add the carbolic acid solution. Instead of using the dry fuchsin and alcohol, 11 cc. 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	3 cc.
Carbolic acid (5 % solution)	20 cc.

If the mixture is not clear, add more of the saturated alcoolic solution of fuchsin drop by drop until when viewed hrough the pipette by transmitted light the liquid is perfectly lear.

ANILINE GENTIAN VIOLET (EHRLICH-WEIGERT)

Saturated alcoholic solution of gentian violet	11 cc.
Absolute alcohol	10 cc.
Aniline water	100 cc.

It is more convenient for each student to prepare one fifth f this quantity.

CARBOLIC THIONINE BLUE (NICOLLÉ)

Thionine blue	1 gram
Carbolic acid	2.5 grams
Distilled water	100 cc.

Filter. Before using, dilute with an equal quantity of distilled vater and filter again.

CARBOLIC GENTIAN VIOLET (NICOLLÉ)

Gentian violet (saturated alcoholic solution)	10 cc.
Carbolic acid (1 % solution)	100 cc.

Mix and filter before storing.

38. Aqueous solutions. Aqueous solutions of methyl violet, entian violet, fuchsin, and the other aniline dyes are prepared y adding 1 cc. of the saturated alcoholic solution of the esired dye to 20 cc. of distilled water. This will impart a ecided color to the liquid, so that in a pipette it will be barely ransparent.

The true aqueous solutions are made by dissolving the dyes 1 water, but these are weak and not so effective as those pre- ared from the alcoholic solutions. These solutions deteriorate 1 a short time. The carbol fuchsin and alkaline methylene lue will keep a little longer, but they require filtering occa- ionally.

39. Making aniline water. Aniline water is a saturated aqueous solution of aniline oil. It is prepared by adding 1 cc. of aniline oil to 20 cc. 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.

40. 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.)

GRAM'S SOLUTION (LUGOL'S)

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

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

41. The general principle underlying the separation of bac-ria by means of plate and roll cultures is to dilute the sub-ance containing the bacteria so that the individual organisms ill be separated from each other by an appreciable distance id then fixed in a solid medium where each organism can ultiply into a growth or colony without coming in contact ith any other organism or colony. For this purpose agar id gelatin are used. Originally Koch employed a rectangu-r piece of glass for holding the layer of medium, and pro-cted it from contamination by putting it under a bell jar. ater Esmarch introduced the "roll-culture" method, which as extensively followed until the Petri dishes were intro-uced. Since that time the latter have been largely used in lace of the Koch plate and Esmarch tube. On this account ie plate cultures of to-day are usually made in Petri dishes. he roll culture is also used.

Plate cultures are employed for two distinct purposes: 1) to isolate bacteria in order to obtain pure cultures from ie isolated colonies; and (2) to determine how many bacteria iere are present in a given quantity of a liquid such as water, ilk, or blood. In this exercise the object is to separate the icteria to obtain isolated colonies. For quantitative work, e Exercise LV.

42. Work for this exercise. Make a series of 3 agar plates, ie of 3 gelatin plates, and one of 3 gelatin roll cultures Esmarch rolls) from the bouillon culture of *B. coli communis* 19). Place the agar plates in the incubator and the gelatin ates and rolls in a locker for that purpose.

Reëxamine all the cultures made in previous exercises and ld 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.

43. Making agar plate cultures. Take 3 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 labeling, number the tubes 1, 2, and 3.

Place 3 sterilized Petri dishes on the leveling 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 one loopful of the bouillon culture, place it in agar tube 1 and mix by carefully shaking. 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. Place the label near the edge of the cover. The Faber pencil for marking glass may be used instead of the gummed label. In making the dilutions it is important that the wire loop should be flamed after making each transfer.

44. Making gelatin plate cultures. These are prepared precisely as the agar plates, with these exceptions: (1) the

atin is liquefied at a temperature of 45° C; (2) the plates en made are to be kept in the locker the same as the gelatin b cultures; (3) in hot weather it is sometimes necessary put a piece of ice in the reservoir under the glass plate on e leveling tripod to congeal the gelatin.

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

45. Making Esmarch roll cultures. For this purpose gela- is ordinarily used. Agar does not adhere readily to the les of the tubes, but is sometimes used. Take the desired mber of large tubes of gelatin, liquefy, inoculate, label, and mber the dilutions as in making gelatin plate cultures. ace a block of ice about 6 inches long in an agate-iron or ss tray. Melt a slight, nearly horizontal groove in the ice th a test tube containing hot media or water. The inocu- ed tubes are tipped and rolled so that the liquid gelatin oistens the inside of the tube to within about a centimeter the plug. Then roll the tube rapidly in the groove on the e until the medium becomes solid. The gelatin should not me in contact with the plug. In rolling the tube the plugged d should always project beyond the ice. (See illustration in ct-books.)

EXERCISE IX

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

46. 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.

47. 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 (§ **35**) the appearance of the bacteria in these preparations.

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

48. 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*) on the surface, (*b*) in the depth of the medium.

Indicate their shape (see § **51**). Are the edges sharply defined? Is the margin even or irregular? Give their size (diameter in millimeters) and indicate their color (determine shade from a color chart[1]) 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.

49. Estimating the number of colonies on plates. If the number of colonies is not large (not exceeding 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 areas 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 Jeffers[3] is more suitable. It consists of a disk about 20 cm. in diameter, divided into areas of a square centimeter each. Place the Petri dish over the disk, taking care that it is centered.

[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 (10 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.

50. 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 charge the needle again from the colony. If the plate is to be rejected, the cover may be entirely removed in the beginning. The newly inoculated tubes or subcultures should be labeled 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. Single particles of dust often carry two or more bacteria.

51. Chester's terminology for description of colonies.

1. Form of colonies. Plate culture.

Punctiform: dimensions too slight for defining form by naked eye, minute, raised, semispherical.
Round: of a more or less circular outline.
Irregular.
Elliptical.
Fusiform: spindle-shaped, tapering at each end.
Cochleate: spiral or twisted like a snail shell.

Amœboid: very irregular, streaming.

Mycelioid: a filamentous colony with the radiate character of a mold.

Filamentous: an irregular mass of loosely woven filaments.

Floccose: of a densely woolly structure.

Rhizoid: of an irregular branched, rootlike character, as in *Bact. mycoides.*

Conglomerate: an aggregate of colonies of similar size and form.

Toruloid: an aggregate of colonies like the budding of the yeast plant.

Rosulate: shaped like a rosette.

. Detailed character of surface.

Smooth: surface even, without any of the following distinctive characters.

Alveolate: marked by depressions separated by thin walls, so as to resemble a honeycomb.

Punctate: dotted with punctures like pin pricks.

Bullate: like a blistered surface, rising in convex prominences, rather coarse.

Vesicular: more or less covered with minute vesicles, due to gas formation; more minute than bullate.

Verrucose: wartlike, bearing wartlike prominences.

Squamose: scaly, covered with scales.

Echinate: beset with pointed prominences.

Papillate: beset with nipple- or mamma-like processes.

Rugose: short, irregular folds, due to shrinkage of surface growth.

Corrugated: in long folds, due to shrinkage.

Contoured: an irregular but smoothly undulating surface, like the surface of a relief map.

Rimose: abounding in chinks, clefts, or cracks.

Internal structure of colony (microscopic).

Amorphous: without definite structure as below specified.

Hyaline: clear and colorless.

Homogeneous: structure uniform throughout all parts of the colony.

Homochromous: color uniform throughout.

Granulations or blotchings.

Finely granular.

Coarsely granular.

Grumose: coarser than the preceding, a clotted appearance, particles in clustered grains.

Moruloid: having the character of a *morula* segmented, by which the colony is divided into more or less regular segments.

Clouded: having a pale ground, with ill-defined patches of a deeper tint.

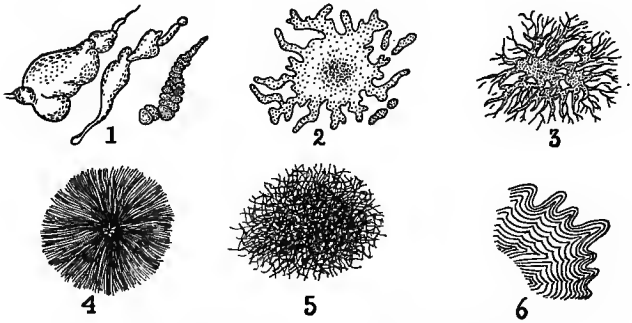


FIG. 4. Types of colonies: 1, cochleate; 2, amœboid; 3, rhizoid; 4, mycelioid; 5, filamentous; 6, curled structure.

4. Colony marking or striping (surface).

Reticulate: in the form of a network like the veins of a leaf.

Areolate: divided into rather irregular or angular spaces by more or less definite boundaries.

Gyrose: marked by wavy lines indefinitely placed.

Marmorated: showing faint, irregular stripes, or traversed by veinlike markings as in marble.

Rivulose: marked by lines, like the rivers of a map.

Rimose: showing chinks, cracks, or clefts.

Filamentous: as already defined.

Floccose: composed of filaments densely placed.

Curled: filaments in parallel strands, like locks or ringlets, as in agar colonies of *Bact. anthracis.*

5. Edges of colonies.

Entire: without toothing or division.
Undulate: wavy.
Repand: like the border of an open umbrella.
Erose: as if gnawed, irregularly toothed.
Lobate.
Lobulate: minutely lobate.
Auriculate: with earlike lobes.
Lacerate: irregularly cleft, as if torn.
Fimbriate: fringed.
Ciliate: hairlike extensions, radiately placed.
Tufted.
Filamentous: as already defined.
Curled: as already defined.

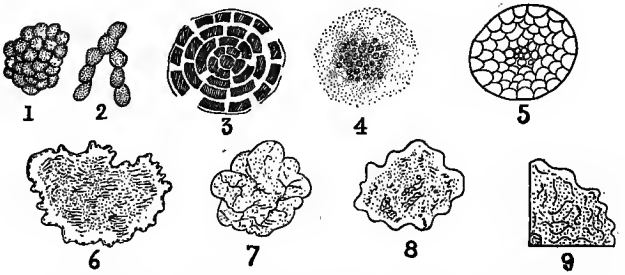


FIG. 5. Structure of colonies: 1, conglomerate colony; 2, toruloid colony; 3, alveolate structure; 4, grumose in center; 5, moruloid; 6, clouded; 7, reticulate; 8, marmorated; 9, gyrose.

6. Optical characters (after Shuttleworth).

Transparent: transmitting light.
Vitreous: transparent and colorless.
Oleaginous: transparent and yellow; olive to linseed oil colored.
Resinous: transparent and brown, varnish or resin colored.
Translucent: faintly transparent.
Porcelaneous: translucent and white.
Opalescent: translucent, grayish white by reflected light, smoky brown by transmitted light.

Nacreous: translucent, grayish white, with pearly luster.
Sebaceous: translucent, yellowish or grayish white.
Butyrous: translucent and yellow.
Ceraceous: translucent and wax colored.
Opaque.
Cretaceous: opaque and white, chalky; dull, without luster.
Dull: without luster.
Glistening: shining.
Fluorescent.
Iridescent.

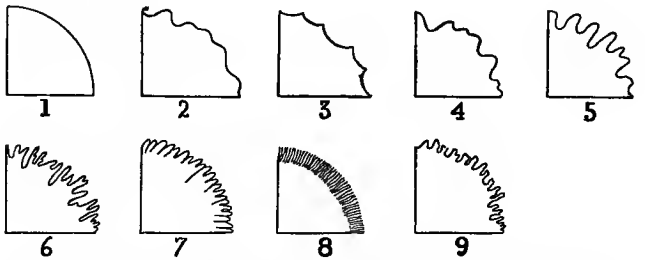


FIG. 6. Character of borders of colonies: 1, entire; 2, undulate; 3, repand; 4, lobate-lobulate; 5, auriculate; 6, lacerate; 7, fimbriate; 8, ciliate; 9, erose.

EXERCISE X

THE PREPARATION OF CERTAIN DIFFERENTIAL AND SPECIAL MEDIA

52. 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.

53. 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, 2 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.

54. 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 from 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), place them in test tubes of the proper size (large or small according to size of cutter used), and add about 1 cc. 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.

55. Preparation of milk for a culture medium. It is better that the cream be removed from the milk before it is used. To do this the fresh milk (about 100 cc.) is placed in a beaker and set in the ice box 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 that may be left. The reaction should be tested, and if strongly acid, should be rejected or made 1.5% acid to phenol-phthalein by the addition of n/10 sodium hydrate. Distribute the skimmed milk in small test tubes (5 cc. 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.

56. 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.

57. Preparation of glucose (grape sugar) agar. Take 30 cc. of agar previously prepared (§ **18**), liquefy, and add 1% glucose while hot. After thoroughly mixing, distribute it in small sterile test tubes. Sterilize, wipe, label, and store the same as ordinary agar.

58. Preparation of glycerin agar. Take 50 cc. of the agar previously prepared and add 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.

59. Preparation of glucose bouillon. This is used in the fermentation tube. Take 100 cc. of sugar-free peptonized bouillon (§ **62**) 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 5 cc. 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, labeled, and placed in the locker until needed for use.

60. Preparation of lactose bouillon. This is prepared by adding 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 and thoroughly mixed in a few cubic centimeters of the bouillon, distribute in fermentation tubes and small test tubes, sterilize, label, and store the same as the glucose bouillon.

61. 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.

62. 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*) 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, distributed in tubes or flasks as desired, and sterilized the same as bouillon (§ **12**).

63. Preparation of acid agar. This is prepared in the same manner as ordinary agar (§ **18**), with the exception that the bouillon should have a reaction of + 2.5.

64. Preparation of acid glycerin agar. Add 5% glycerin to acid agar before sterilizing it.

65. Preparation of acid glycerin bouillon. This is prepared either as ordinary bouillon (§ **12**) or as sugar-free bouillon (§ **62**), with the exception that it is titrated to a reaction of + 2.5, and the addition of 5% c.p. glycerin.

66. 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 to 7 cc. 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 slaughterhouse.

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

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 4 consecutive days.

67. Preparation of Loeffler's blood serum. This consists of 1 part neutral bouillon (prepared from meat) containing 1% grape sugar and 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 necessarily be longer than for the pure blood serum. When it is to be used for the cultivation of diphtheria organisms it can be set at a much higher temperature (80° to 90° C.). Label and store.

68. Preparation of egg medium. The whole egg is preferable. Carefully break the shell of the required number (3, 6, or more) of fresh eggs, drop the entire contents into a clean dish, and beat until the yolk and white are thoroughly mixed. Add an equal volume of glycerin bouillon of a reaction + 1.3. Place in tubes, care being taken to avoid bubbles. Sterilize by heating, preferably in a serum water bath at from 75° to 80° C. for from 4 to 5 hours each day for 2 days. After sterilization the tubes, if test tubes, should be sealed. Before using, add a few drops of sterile water or, better perhaps, of 5% glycerin bouillon, to afford sufficient moisture. This is most used at the present time for the cultivation of tubercle bacteria.

69. Preparation of nitrate bouillon. Take peptonized bouillon 100 cc. Add potassium nitrate (0.5 %) 1 gram. Dissolve the nitrate in the bouillon, put in tubes, and sterilize the same as bouillon.

The nitrate of sodium or ammonium may be substituted for that of potassium. The salt may be added in the proportion of from 0.1 to 1% to meet special demands.

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

70. Grouping of culture media. For convenience in reference and assignment of media, the culture media most commonly

employed in studying and differentiating species of bacteria have been arranged arbitrarily in groups. Large test tubes containing agar and gelatin for making plate cultures are not included in these groups.

Group A. Media commonly used.

A tube of bouillon.
A tube of sugar-free bouillon.
A tube of agar.
A tube of gelatin.
A tube of milk.
A tube of litmus milk.
A tube of potato.

Group B. Media favorable to determine the power of bacteria to ferment the sugars with the formation of acids.

A tube of bouillon containing 1 % grape sugar (dextrose, glucose).
A tube of bouillon containing 1 % milk sugar (lactose).
A tube of bouillon containing 1 % cane sugar (saccharose).

Group C. Media favorable for determining the production of gas.

A tube of agar containing 1 % grape sugar.
A tube of agar containing 1 % milk sugar.
A tube 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.

Fermentation tube containing 1 % grape-sugar bouillon.
Fermentation tube containing 1 % milk-sugar bouillon.
Fermentation tube containing 1 % cane-sugar bouillon.

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 (in small tubes).
Acid glycerin agar (in small tubes).
Acid glycerin bouillon (in small tubes).
Blood serum (dog) solidified at 70° to 75° C. (ground-glass-capped tubes).
Loeffler's blood serum (usually small tubes).
Egg medium (Dorset).

EXERCISE XI

INOCULATING SPECIAL MEDIA AND EXAMINING CULTURES

71. Work for this exercise. Inoculate, from a culture furnished of *B. proteus vulgaris*,[1] a tube of potato, one of milk, one of litmus milk, one of glucose agar, a fermentation tube and a test tube each of glucose, lactose, and saccharose bouillon. Label each and place in the incubator.

Stain a preparation with alkaline methylene blue, another with carbol fuchsin, and a third with an aqueous solution of gentian violet from the bouillon and agar cultures (§ **47**). Make a careful comparison of the preparations and note any difference in the appearance of the bacteria or in the 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 gentian violet (§ **38**).

72. 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.

73. The use of media containing the sugars. The sugars are employed as tests to determine whether or not 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,

[1] Or any other gas-producing bacillus.

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 the power of the organism to produce gas, use only the agar tubes.

2. If to determine the power of the organism to ferment sugars, producing acids, use only 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, WITH A STUDY OF THE GAS PRODUCTION

74. 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.

The knowledge of the powers of a given species of bacteria to produce gas when grown in a medium containing sugar is also 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 with 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 *B. suipestifer* and *B. typhosus*.

75. 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

a stained (carbol fuchsin) cover-glass preparation. Describe the appearance of the bacteria (§ **35**). Examine the fermentation tubes and indicate the quantity of gas.

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

Make a stained cover-glass preparation from the milk culture. Stain with carbol fuchsin.

76. 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 have been changed, and 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, its chemical reaction as indicated by litmus paper (§ **27**), 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 (§ **27**).

(*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 in (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.

In studying the cultures in the fermentation tubes 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.

77. Determination of 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.

78. 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, covering it with the thumb and again bringing the gas to the open bulb, holding it close to a flame, and removing 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 in the closed branch = 5 cm. The amount absorbed (CO_2) = 2 cm. The remaining gas, or 3 cm., = H. The ratio of CO_2 to H is, therefore, as 2 : 3, or CO_2 : H : : 2 : 3.

79. Making cover-glass preparations from milk cultures. Spread as thin a film of the milk culture as possible 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 centimeters 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 XIII

THE CLASSIFICATION OF BACTERIA AND THE EXAMINATION OF CULTURES

80. 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; but this is true of any logical system. The restoration of the genus *Bacterium* and the assigning to it of all nonmotile, rod-shaped organisms change 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*.

81. Work for this exercise. Read the references on the morphology and classification of bacteria.

Reëxamine all the cultures previously made and make careful notes of any changes that have occurred in their appearance.

Measure the gas in the fermentation tubes and determine the ratio of CO_2 to H. See page 50

Discard all the cultures made and clean the tubes and Petri dishes [§ **3**, (*f*)].

Inoculate a tube of bouillon and one of slant agar (from cultures furnished) with each of the following genera of bacteria: (1) a streptococcus, (2) a micrococcus, (3) a sarcina.

82. Migula's classification of bacteria.

FAMILIES

I. Cells globose in a free state, not elongating in any direction before division into 1, 2, or 3 planes	1. COCCACEÆ
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, nonmotile, or motile by means of flagella	2. BACTERIACEÆ
2. Cells crooked, without sheath .	3. SPIRILLACEÆ
3. Cells inclosed in a sheath . . .	4. CHALMYDOBACTERIACEÆ
4. Cells destitute of a sheath, united into threads, motile by means of an undulating membrane .	5. BEGGIATOACEÆ

1. GENERA BELONGING TO THE FAMILY COCCACEÆ

Cells without organs of motion.	
a. Division in 1 plane	1. *Streptococcus*
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. GENERA BELONGING TO THE FAMILY 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. GENERA BELONGING TO THE FAMILY SPIRILLACEÆ

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 to 3, polar flagella . . . . . 2. *Microspira*
  - (2) Cells with polar flagella, in tufts of from 5 to 20 . . . . . 3. *Spirillum*

Cells flexuous . . . . . 4. *Spirochæta*

4. GENERA BELONGING TO THE FAMILY CHLAMYDOBACTERIACEÆ

I. Cell contents without granules of sulphur.
- *a.* Cell threads unbranched.
  - (1) Cell division always only in 1 plane 1. *Streptothrix*
  - (2) Cell division in 3 planes previous to the formation of conidia.
    - (*a*) Cells surrounded by a very delicate, scarcely visible, sheath (marine) . . . 2. *Phragmidiothrix*
    - (*b*) Sheath clearly visible (in fresh water) . . . . 3. *Crenothrix*
- *b.* Cell threads branched (pseudobranches) . . . . . 4. *Cladothrix*

II. Cell contents containing sulphur granules . . . . . 5. *Thiothrix*

5. GENERA BELONGING TO THE FAMILY BEGGIATOACEÆ

Only one 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.

EXERCISE XIV

THE MORPHOLOGY OF STREPTOCOCCUS, MICROCOCCUS, AND SARCINA

83. 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 centered 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.

84. Work for this exercise. Carefully describe each of the cultures made in Exercise XIII.

Prepare and examine a hanging-drop preparation from each of the 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 each culture and stain with an aqueous solution of methyl violet (§ **38**). 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 the notes.

Inoculate a tube of bouillon and one of slant agar from cultures (furnished) of each of the following genera of bacteria: (1) a bacterium, (2) a bacillus (3 cults), (3) a spirillum.

(Select species of bacilli that illustrate slow and rapid motility and spores.)

Inoculate a tube of bouillon and one of slant agar with *B. Subtilis* for Exercise XVI.

Inoculate a tube of slant agar with a bacillus (some member of the colon or intermediate group is preferable) for flagella staining for Exercise XVI.

EXERCISE XV

THE MORPHOLOGY OF BACTERIUM, BACILLUS, AND SPIRILLUM

85. The organisms which are to be studied in this exercise exhibit in addition to the rod-shaped bodies (bacterium and bacillus) the essential morphological variations of these genera, viz. number of flagella and spores. The staining of the organs of locomotion (flagella) and the spores will be taken up in another exercise.

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

Measure carefully with the filar micrometer the length and thickness of 5 individual bacteria in one of the stained preparations. Record the measurements in microns (written μ).

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

State fully in the notes the generic characters of the genera Streptococcus, Micrococcus, Sarcina, Bacillus, Bacterium, and Spirillum.

Prepare water suspensions for staining flagella (§ **91**).

87. Making drawings of bacteria with a definite magnification. In measuring the bacteria we obtain the dimensions in microns or in units of $\frac{1}{1000}$ of a millimeter. In making a drawing, therefore, showing them magnified 1000 diameters, it is simply necessary to represent each micron by a millimeter. Thus, if the organism is 2.5 μ in length and 1 μ broad, and 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 XVI

STAINING SPORES AND FLAGELLA

88. 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 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.

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

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.

89. Work for this exercise. Examine and carefully describe the cultures of *B. subtilis* made in Exercise XIV. *omit*

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 without 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. *Note Spor*

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

Clean about 10 cover glasses after the special method for flagella staining (§ 4). Make about 5 films on these from the water suspension prepared at the last exercise and stain for flagella. Use Johnston and Mack's modified method, with Loeffler's mordant and stain. The films should not be heated either before or during the staining process, but if it does not succeed, Loeffler's process may be tried.

90. 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 heat again. 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 cc. of 1% solution of sulphuric acid or 95% alcohol. After about ½ 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, counterstain 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.

91. Methods for staining Flagella — Johnston and Mack's modified method. *Preparation of films.* Make a culture on slant agar of the organism to be stained and incubate for from 18 to 24 hours. Prepare a tube with 6 to 8 cc. of sterile water, and keep it in the incubator until it is of the same temperature. With a sterile platinum loop scrape away some of the growth from the agar surface, using care not to remove any of the agar, and rinse it off carefully in the tube of water previously prepared for this purpose. There should be enough to impart to the water a faint cloudiness. This should be done in a warm room and with considerable care. Replace this tube in the incubator and the bacilli will distribute themselves quite evenly through the water, though any clumps or masses will settle to the bottom. The tube is left in the incubator for from 2 to 3 days before preparing the films. Place a tray of properly prepared cover glasses in the incubator to warm ; then, still in the warm room, with a platinum loop put a drop or two from the tube on each cover (§ 4). Replace the tray in the incubator until the water has evaporated, when the films are ready to stain. The films require no fixing other than by the mordant.

With a pipette apply to a film all the mordant the cover glass will hold, allow it to act 2 or 3 minutes without heating, and rinse thoroughly in clean water ; apply the stain in the same manner, allow it to act 2 or 3 minutes without heating, rinse well, and examine in water, or dry and mount in balsam.

All these solutions should be freshly prepared. Considerable care in washing after the mordant will be well repaid, as it insures a cleaner background.

Around the edge where the drop was applied to the cover glass a heavy line of bacteria will be found, and if the right amount

of culture was added to the water, many will be found scattered within the ring, some of them isolated so they can be easily studied. The flagella should appear as fine, hairlike appendages radiating from the bacteria. A little practice with this will enable one to make good preparations altogether free from background.

92. Mordants and stains. Different mordants and staining solutions may be used. Those of Loeffler or Pitfield give the most uniform and satisfactory results.

LOEFFLER'S MORDANT

Twenty per cent aqueous solution tannic acid	10 cc.
Saturated aqueous solution iron sulphate	5 cc.
Saturated alcoholic solution basic fuchsin	1 cc.

Mix, let stand 2 or 3 hours, and filter.

Tannic acid solution should be freshly prepared, but the iron sulphate solution is better if it stands until it begins to turn brownish by oxidation, but it should not be too old. If, when this mordant is used, it gives a precipitate, filter again. When properly prepared it should have much the same color as a solution of hematoxylin.

LOEFFLER'S STAIN (ZIEHL'S CARBOL FUCHSIN)

Saturated alcoholic solution basic fuchsin	3 cc.
Five per cent carbolic acid	20 cc.

If not clear, add fuchsin solution drop by drop until it clears, then filter.

PITFIELD'S MORDANT

Ten per cent aqueous solution tannic acid	10 cc.
Saturated aqueous solution mercuric chloride	5 cc.
Saturated aqueous solution potassium alum	5 cc.
Ziehl's carbol fuchsin	5 cc.

Mix, let stand 2 or more hours, and decant clear fluid or filter.

PITFIELD'S STAIN

Saturated aqueous solution potassium alum	10 cc.
Saturated alcoholic solution gentian violet	2 cc.

Mix, let stand 2 or more hours, and filter.

Saturated alcoholic solution of methyl violet, basic fuchsin, or methylene blue can be substituted for gentian violet in the above formula with equally good results.

93. Staining the flagella by Loeffler's method. Place 2 loopfuls of sterilized distilled water or normal salt solution on the center 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.

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 Loeffler's mordant.

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 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.

EXERCISE XVII

STAINING TUBERCLE BACTERIA (BACILLI)

94. The stained tubercle bacteria possess, because of the layer of fatty acids covering them, 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 largely depended upon in making a differential diagnosis.

95. Work for this exercise. Make 4 cover-glass preparations from a culture of tubercle (furnished). Stain 2 of them for tubercle bacteria and carefully describe the appearance of the bacteria and illustrate with a few drawings.

Counterstain 2 of the preparations after Gabbett's method.

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

For the next exercise liquefy 2 large tubes of agar and 2 of gelatin and pour them into Petri dishes. After the medium has solidified remove the covers of the Petri dishes and expose one of each to the air for 5 minutes and one of each for 10 minutes. Return the covers and place the agar plates in the incubator and the gelatin ones in the locker. When these plates are examined in the next and subsequent exercises there will be a colony for each bacterium that fell upon the medium from the air. It will be necessary to look out for impure or mixed colonies, as two or more organisms may have fallen together.

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

96. 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 3% solution of HCl in 95% alcohol for from ¼ 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 counterstain, 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 counterstain is of no value in preparations made from pure cultures or for simple diagnostic purposes. When a counterstain is desired Gabbett's decolorizing and counterstaining solution is very convenient.

GABBETT'S SOLUTION

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

After staining with the carbol fuchsin treat the preparations with this mixture until the film has a faintly bluish tint. This solution decolorizes and counterstains at the same time. This organism, like some other pathogenic bacteria, takes the Gram stain.[1]

[1] See Novy's *Laboratory Work in Bacteriology*, p. 289, for a list of such organisms.

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.

Fontes method of staining tubercle bacteria. (*a*) Stain the preparation with ordinary carbol fuchsin for from 5 to 10 minutes, applying heat.

(*b*) Wash in tap water.

(*c*) Stain about 2 minutes with carbol gentian violet.

(*d*) Treat with Lugol's solution until no more metallic mirrors are formed (decolorization is more easily effected if at this point the preparation is blotted thoroughly).

(*e*) Treat with acetone alcohol (equal parts of alcohol and acetone). Wash until the stain ceases to wash out.

(*f*) Wash in distilled water.

(*g*) Counterstain with methylene blue.

Cent. f. Bakt. 1. Abt. Orig., Feb. 26, 1909.

Herman's method of staining tubercle bacteria. Stain the preparation for from 5 to 10 minutes, using heat. Herman employs a stain composed of a mixture of 3 parts of a 1% ammonium carbonate solution and 1 part of a 3% crystal violet solution in 96% alcohol. The preparations are stained in this from 5 to 10 minutes, using heat.

They are then decolorized for several seconds in 10% nitric acid, and then in 96% alcohol.

The tubercle bacteria will be stained a brilliant violet.

Cent. f. Bakt. 1. Abt. Orig., Bd. 60. S. 600.

EXERCISE XVIII

A STUDY OF CERTAIN SAPROPHYTIC BACTERIA

97. It is desirable to have a definite knowledge concerning the characters and properties of the commonly encountered species and groups of saprophytic bacteria. It is likewise important to understand the method of identifying species. For these reasons a few exercises on saprophytic bacteria, especially from air, milk, and water, have been introduced.

98. Work for this exercise. Examine and carefully describe the 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. Make a microscopic examination (hanging-drop) of the bacteria in one of each of the different kinds of colonies and determine its genus.

Make for examination in the next exercise a series of 2 plate cultures in gelatin and one of 2 plates in agar from a sample of milk furnished. The milk will be either freshly drawn in sterile flasks or samples of market milk.

99. Identifying species of bacteria. The genera of bacteria are determined by the morphology. Thus, a spherical organism is a *micrococcus*; a motile, rod-shaped one is a *bacillus*; and a nonmotile, rod-shaped one is a *bacterium*. Each genus has a large number of species. This requires some method by which bacteria which look exactly alike under the microscope may be differentiated, provided they are different. This method consists in the study of the growth of these bacteria on the different media and possibly their effect upon small animals. For example, the *B. typhosus* and *B. coli* look so nearly alike that one could not be sure of a difference microscopically: *B. coli* coagulates milk, *B. typhosus* does not; *B. coli* produces gas in glucose media, *B. typhosus* does not. Knowing these properties and having these cultures we could readily tell the

one from the other. To identify species, therefore, one must compare the cultural characters of the organism in question with the description of the species already known. As new media are constantly being introduced, one often finds that the descriptions given in text-books and manuals of bacteriology are very brief and, as compared with modern requirements, insufficient to identify the species. This has resulted in the listing of a very large number of species that are very difficult, if not impossible, to identify. With pathogenic bacteria the somewhat specific action on experimental animals affords much aid in their identification. For further explanations, see text-books. Matzuschita's *Bacteriologische Diagnostik* is especially helpful in diagnosing species.

The descriptive chart of the Society of American Bacteriologists for the identification of species is very convenient for recording the morphological, cultural, physical, chemical, and pathogenic properties of bacteria.

9.3 Dilution.
9.1

EXERCISE XIX

A STUDY OF BACTERIA IN MILK

100. 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 the bacteria in ordinary market milk should be studied, though briefly.

101. 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 colony and determine its genus.

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

Inoculate group A of media from a culture of *B. prodigiosus* furnished.

Make, for examination at the next and following exercises, 3 gelatin plates from a sample of water furnished (unfiltered creek or well water), using for each culture the quantity designated by the instructor. The quantity will depend upon the condition of the water.

EXERCISE XX

A STUDY OF BACTERIA IN WATER

102. It is important to know something of the bacterial contents of water as it is found in wells and streams, and to compare the bacteria ordinarily found in water with those present in freshly drawn milk. It will be observed that the normal bacterial flora of water and of milk are quite different.

103. Work for this exercise. Carefully examine and describe the cultures made from the colonies on the milk plates in the last exercise.

Examine and describe the colonies on the plate cultures made from water. Determine the number of colonies and approximately the number of each kind of colony on the plate.

Examine microscopically the bacteria from one of each kind of colony and determine the genus.

Inoculate a tube of milk and one each of bouillon, agar, and gelatin from each of 3 different colonies, if there are as many. In later exercises examine these cultures and compare them with those made from colonies of milk bacteria.

Examine and describe the cultures of *B. prodigiosus*.

Inoculate groups A and B of media from a culture of *Ps. fluorescens liquefaciens* furnished.

EXERCISE XXI

A STUDY OF CERTAIN PYOGENIC BACTERIA

104. 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* and *Ps. pyocyaneus*, are frequently found as the apparent cause of suppuration. As it is impossible to study more than a few of these species, two of the most common and one of the more rarely encountered organisms in suppurating wounds and abscesses are chosen for special study.

105. Work for this exercise. Inoculate a tube of each medium in groups A and B from each of the cultures of *Streptococcus pyogenes* and *Micrococcus* (*Staphylococcus*) *pyogenes* and *Ps. pyocyaneus* which will be furnished.

Describe the cultures of *Ps. fluorescens liquefaciens* and examine the bouillon culture microscopically in a hanging-drop and in a stained preparation.

Examine and describe the milk, bouillon, agar, and gelatin cultures made from the colonies from milk and water plates.

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

Give in the notes a definition of each of the following terms: saprophytic, parasitic, pathogenic, and pyogenic bacteria. See text-books.

EXERCISE XXII

PYOGENIC BACTERIA (*continued*)

106. *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 *Ps. fluorescens liquefaciens* and its varieties, which frequently appear in water.

107. Work for this exercise. Examine and carefully describe the cultures made in Exercise XXI. Note especially the growth on the agar, gelatin, and 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 cover-glass preparations the bacteria from 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 XXIII a tube of each medium in groups A and B from a culture (furnished) of *Bact. Anthracis.*

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

Include in the notes the names of the different forms of *Micrococcus pyogenes* and a classification of streptococci.

EXERCISE XXIII

BACTERIUM ANTHRACIS

108. Anthrax is a disease affecting cattle and man. *Bact. anthracis* is interesting because of its spores, which are very resistant to disinfectants. Because of its striking morphology it can be differentiated microscopically in fresh tissues. In tissues some hours after death there is a putrefactive organism that resembles it morphologically. *Bact. anthracis* can be diagnosed in cultures.

109. 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.

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

Make a series of 2 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.)

In notes state whether or not a microscopic examination of tissues is sufficient to make a diagnosis of anthrax.

Inoculate a tube of glycerin agar, one of egg medium, and one of glycerin bouillon from a culture of avian tubercule bacteria for study in Exercise XXVIII.

110. 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 being taken that the film of tissue is thin. Allow this to dry in the air, after which pass the cover glass, film up, 3 times through the flame to fix the tissue to the glass. It can be stained the same as the cover-glass preparations

from the cultures. When carbol fuchsin is used for staining, the preparation should be wet before applying the stain. 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. With the platinum loop place a drop of blood 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 made.

EXERCISE XXIV

BACTERIUM ANTHRACIS (*continued*)

111. Work for this exercise. Reëxamine all of the cultures of *Bact. anthracis* and describe any changes in their appearance which may have taken place.

Stain a few cover-glass preparations according to Gram (§ **40**).

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

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 Loeffler's blood serum and glycerin agar with *Bact. diphtheriæ* from a culture furnished, for study at the next exercise.

EXERCISE XXV

BACTERIUM DIPHTHERIÆ

112. 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 throats of people suffering with diphtheria, and often in the throats and noses of those who have been exposed to it. These are designated as "germ cases." Ordinarily it is not found 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 made use 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.

113. Work for this exercise. Examine microscopically, in hanging-drop and 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. The preparation should be stained for fully 5 minutes and then decolorized for a few seconds in 95% alcohol.

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

114. Neisser's method of staining diphtheria bacteria. Neisser has recommended the following method of staining, in which 2 solutions are employed; viz.,

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

2. Two grams of vesuvin are dissolved in 1 litre of boiling distilled water and filtered.

The cover-glass preparations are stained in (1) for from 2 to 5 seconds, washed in water, and then stained in (2) for from 5 to 10 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]

[1] Clinically, *Bact. 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 very closely in its morphology the Klebs-Loeffler bacillus. It is also to be distinguished from the xerosis bacillus isolated by Neisser and from Hoffman's bacillus. For detailed descriptions of these organisms, see text-books.

EXERCISE XXVI

BACTERIUM MALLEI

115. This organism grows most characteristically on potato and somewhat feebly in the other media heretofore used. It develops readily on acid agar and on acid glycerin agar and in acid glycerin bouillon. For this reason it is not inoculated into all the media. In diagnosing glanders it is customary to inoculate guinea pigs with the suspected material. 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.

116. 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, one of bouillon, and one of acid glycerin bouillon from a culture of *Bact. mallei* (furnished). (The special media here introduced will be furnished by the instructor.)

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 to which the organisms take the stain.

Reëxamine the cultures of *Bact. diphtheriæ* and complete the notes on the same.

EXERCISE XXVII

BACTERIUM MALLEI (*continued*)

117. Work for this exercise. Examine and carefully describe all the cultures of *Bact. mallei.*

Make 2 cover-glass preparations from the acid glycerin agar and from the bouillon cultures, and stain one of each with alkaline methylene blue and one with carbol fuchsin. Describe the bacteria and make a drawing of a few of them. Preserve one preparation. Keep the cultures and reëxamine them at each of the three following exercises. Note especially the character and color of the growth on the potato and in the acid glycerin bouillon.

Examine carefully the lesions produced by the inoculation of *Bact. mallei* in a male guinea pig (Straus' method of diagnosing glanders).

EXERCISE XXVIII

BACTERIUM TUBERCULOSIS

118. The tubercle bacterium does not grow readily on the ordinary media. For its cultivation blood serum, egg medium, glycerin agar, or bouillon containing from 5 to 7% glycerin is ordinarily used. Formerly it was with much difficulty that it was made to grow from lesions of 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 Part II, Diagnosis of Tuberculosis). It grows very slowly, and it is necessary that the temperature should be kept, without variation, at about 37° C. The avian variety grows much more readily on glycerin agar, egg medium, serum, and in glycerin bouillon. 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.

119. Work for this exercise. Examine and carefully describe the appearance of the cultures of the tubercle bacterium (human or bovine variety) on glycerin agar and in glycerin bouillon furnished.

Make 2 cover-glass preparations from the cultures furnished for that purpose and stain them with carbol fuchsin (§ **96**).

Make 4 cover-glass preparations from tuberculous sputum and stain for tubercle bacteria. It is often desirable to counterstain the specimens from sputa. Stain two of them by Gabbett's method and two with carbol fuchsin, and decolorize without

counterstaining. (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 acidulated alcohol or a 10% solution of sulphuric acid, and stain one by Gabbett's method.)

Examine the cultures of avian tubercle bacteria and describe their appearance. Stain and examine a few preparations from one of the cultures.

Indicate in the notes the essential differences between human, bovine, and avian tubercle bacteria respecting (1) morphology, (2) cultural properties, and (3) pathogenesis. References.

Measure the tubercle bacteria in one of the preparations and make a drawing showing a few of them magnified 1000 diameters.

Inoculate the media in groups A and B with the culture furnished of *Bact. suisepticus* or some other member of the septicemia hemorrhagica group. (avisepticus - pasteurella)

120. 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, and then to dry, fix, and stain as with the cover glasses. The water is dried off by using filter or blotting paper, and the preparation is 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.

EXERCISE XXIX

BACTERIUM SEPTICEMIÆ HEMORRHAGICÆ GROUP

121. 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 septicemia (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. The diseases caused by these organisms are known as the *Pasteurelloses*, i.e. due to the organisms belonging to the genus *Pasteurella* of Trevisan.

122. Work for this exercise. Carefully examine and describe the cultures made in Exercise XXVIII.

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

Describe the appearance of the bacteria and make from one preparation a drawing of a few of them magnified 1000 diameters.

Preserve a preparation to accompany notes.

Measure a few of the bacteria with the filar micrometer and record the measurements.

Make, stain, and examine a cover-glass preparation from an organ and the blood of a rabbit which has died from the inoculation with swine-plague bacteria (the rabbit will be furnished by the instructor). Stain the preparations with an aqueous solution of fuchsin. Study the bacteria in these preparations and carefully compare the two.

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

Inoculate groups A and D of media with *Bact. pullorum*.

EXERCISE XXX

BACTERIUM PULLORUM

123. *Bact. pullorum* is the cause of a severe diarrhea (bacillary white diarrhea) of young chickens. It has recently been found to cause a disease usually fatal in adult fowls. It differs very little from *Bact. sanguinarium.* It was originally described by Rettger.

124. Work for this exercise. Examine and describe the cultures of *Bact. pullorum* made in the last exercise.

Examine microscopically in both stained and hanging-drop preparations the bouillon and agar cultures.

Note particularly the action of this organism on milk and the sugar bouillons.

Make the indol test (§ **127**) with the culture in sugar-free bouillon of the organism of the septicemia group studied in the last exercise.

Inoculate a tube of each of the media in groups A and D from a culture of *B. coli* (furnished) for study at the next exercise.

EXERCISE XXXI

BACILLUS COLI

125. 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 *B. suipestifer*, 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 bacilli and also to *B. suipestifer*. It is important that this existing variation be recognized and that the list of properties which characterize *B. coli* 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. This is especially true in water analysis. Many special media have been recommended to differentiate these organisms. The culture assigned approaches very closely to the typical species.

126. Work for this exercise. Describe the appearance of each of the cultures made in Exercise XXX.

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 in each tube and the ratio of CO_2 to H.

Make 2 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 if it is at least 72 hours old. Read the chapter on this organism in the text-books.

Inoculate groups A and D of media with *B. suipestifer*[1] from a culture furnished.

127. The indol (cholera-red) test. Add 1 cc. of 0.01% 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 one more recently made.

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

Ring method. When there is a small quantity of indol it can be detected more readily by the "ring method." Add 1 or 2 cc. of a 25% solution of H_2SO_4, allowing it to run down on the inside of the tube containing the culture. Add 1 cc. of potassium nitrite. If indol is present, a pinkish ring will be observed between the layer of acid in the bottom of the tube and the culture above it.

128. 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 cc.

Dissolve the peptone and salt in the water, distribute it in the tubes (5 cc. each), and sterilize the same as bouillon.

[1] In place of this, *B. enteritidis* or some other member of the intermediate group may be substituted.

EXERCISE XXXII

BACILLUS COLI AND BACILLUS SUIPESTIFER

129. Work for this exercise. Reëxamine the cultures of *B. coli* and note any changes which have occurred in their appearance. Determine the gas formula in the fermentation tubes of the different sugars. Place the milk and litmus-milk cultures in the incubator and reëxamine them later.

Examine carefully and describe the cultures of *B. suipestifer*. Examine microscopically at least one culture. Note especially its motility.

Examine and describe fully the colonies on the gelatin plates. Preserve the plates and examine them in 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* from the intestine of an animal. The intestine will be furnished.

Inoculate for Exercise XXXIII a tube of each medium in groups A and D with *B. typhosus*, and in group D with *B. paracolon*, from cultures furnished.

130. Isolating B. coli 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* 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.

EXERCISE XXXIII

TYPHOID AND PARACOLON BACILLI

131. The bacillus of typhoid fever and *B. suipestifer* resemble each other very closely morphologically and in certain of their cultural characters and biochemic properties. Like *B. coli* each of these organisms has several varieties. Several distinct varieties of *B. suipestifer* have been described.

Certain of the varieties of these species approach each other very closely, while others approach *B. coli* 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-books.

132. 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. Note especially the number of colonies of *B. coli* 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* in these media.

Examine and carefully describe the cultures of *B. typhosus* and *B. paracolon*. Note especially the reaction of the cultures in the fermentation tubes. Examine the bouillon culture of *B. typhosus* and the glucose bouillon culture of *B. paracolon* microscopically in hanging-drop and in stained cover-glass preparations. Describe the appearance of the bacteria.

Give in tabulated form the differential characters of the colon, intermediate, and typhoid groups of bacteria.

Inoculate by Liborius' method (§ 135) 2 tubes of liquid agar, one sugar-free, the other containing glucose, and 2 fermentation tubes, one sugar-free, the other containing 1% glucose bouillon, from a culture of an anaërobic organism furnished. *B. chauveaui*, the cause of black leg, is a fitting organism to use in this study. Place the inoculated tubes in the incubator.

EXERCISE XXXIV

CULTURES OF ANAËROBIC BACTERIA

133. 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 natural gas, such as hydrogen. While certain bacteria, like those of symptomatic anthrax, tetanus, and malignant œdema, require the absence of oxygen, others, like *B. 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.

134. Work for this exercise. Examine and carefully describe the appearance of the anaërobic cultures made in Exercise XXXIII.

With the wire loop remove one of the colonies from the depth of the agar culture and examine it microscopically (1) in a hanging-drop preparation and (2) in a stained cover-glass preparation. Stain with carbol fuchsin. Examine microscopically in similar preparations the bacteria from one of the fermentation tubes; describe their appearance 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.

Inoculate (Liborius' method) 2 tubes of agar, one sugar free, the other containing 1% glucose, and 2 fermentation tubes, one containing sugar-free bouillon, the other 1% glucose bouillon from a culture of *B. tetani* (furnished).

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

135. Culture by 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 an anaërobic organism (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.

136. The fermentation tubes for cultivating 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 XXXV

BACILLUS TETANI

137. The bacilli of tetanus or their spores occur in nature as common inhabitants of the soil — at least they are found in the soil in certain localities. They are believed to be more numerous in certain places where manure has been thrown in abundance. *B. tetani* is anaërobic and consequently must be cultivated according to methods necessary for such bacteria (§§ **135, 136**). 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.

138. Work for this exercise. Carefully examine the 4 cultures of tetanus bacilli made in the last exercise and describe their appearance.

Make a cover-glass preparation from the liquid cultures 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, sterilized, and rejected.

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 quantity for the first tube in the plate series. The saliva from a horse or cow may be collected and substituted for human sputum.

139. 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

cultures made directly from the lesions are usually impure. I have found that pure cultures may often 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 from 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 XXXVI

THE BACTERIA OF THE MOUTH

140. In studying cultures from the throats 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, a bacterium not distinguishable from that of swine plague is present in the upper air passages of a large percentage of healthy animals. In order, however, to isolate these organisms, 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.

141. 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, together with the approximate number of colonies of each.

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

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

142. 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 is 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 in the same manner as is sputum.

EXERCISE XXXVII

IDENTIFYING BACTERIA FROM CULTURES

143. The two cultures of bacteria assigned for identification belong to species already studied, and the student should identify the species of bacteria in them. 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. This exercise affords a good opportunity to begin the use of manuals and text-books in identifying species.

144. 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.

Reëxamine and reject the cultures on hand.

EXERCISE XXXVIII

ISOLATING BACTERIA FROM ANIMAL TISSUES

145. 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 from 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) which has died from some bacterial disease will be provided. 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. From time to time during the course opportunity will be afforded for making cultures from variously diseased animal tissues. Each student will be given opportunity at some time during the course to inoculate one or more animals.

146. Work for this exercise. The experimental animal furnished will be 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, a tube 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, and from the blood and the lymphatic glands.)

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

Make and examine 2 cover-glass preparations from the organ from which the cultures were made. Stain one with alkaline methylene blue and one with 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 of the cover-glass preparations.

147. Making cultures from animal tissues. Heat a platinum spatula to a white heat in a gas flame and scorch the surface of the organ. Flame a pair of fine forceps, tear an opening through the scorched surface, and crush a bit of the tissue underneath it. With the platinum loop take up a loopful of the crushed tissue and inoculate the media with it. 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 XXXIX

IDENTIFYING BACTERIA FROM ANIMAL TISSUES AND THE STUDY OF BACTERIA IN SECTIONS OF TISSUES

148. Work for this exercise. Examine and describe all of the cultures made from the animal tissues.

Examine the bouillon and agar cultures microscopically, both 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.

After the species are identified, state in the notes the facts upon which the identification is made.

149. Examine the sections furnished for bacteria and note especially their distribution in the tissues.[1] 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 of the same species, and note any differences in their appearance which may be detected.

[1] 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 should be able to distinguish bacteria in the lesions which they produce. For this reason a part of an exercise is devoted to the study of bacteria in sections of tissues already stained and mounted. These include tuberculosis, anthrax, typhoid, septicemia, the various pneumonias, etc.

27 Anthrax
256 T. B.
374 Johnes disease

EXERCISE XL

BACTERIOLOGIC EXAMINATION OF PUS AND EXUDATES

150. It is often very desirable for diagnostic purposes to make a bacteriologic examination of the pus from abscesses and the mucopurulent 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, gonorrhea, 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 of the more desirable cases will be made from time to time as they become available. In this exercise such cover-glass preparations will be examined as have been accumulated for this purpose.

151. 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 gonorrheal discharge, stain the cover-glass preparations with an alcoholic solution of eosin and 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.

152. 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 (§ **110**).

Nölin phagocytosis.

1. abscess fistula
2. glanders.
3. Milk with T. R.
4. B. Necrophorus - Look up in text
5. B. abortus. Journal of Inf. Diseases Miss Alice Evans.

EXERCISE XLI

A BACTERIOLOGIC EXAMINATION OF THE SKIN AND FINGER NAILS

153. There is liable to be on or in the skin of the hands and about the finger nails a number of bacteria which resist the ordinary methods of cleansing and which often cause infection. The most important among these are pyogenic streptococci and micrococci. 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 these or other species of bacteria. The work of this exercise is to demonstrate the presence of these organisms on the skin of supposedly sterilized hands.

154. Work for this exercise. Wash the hands thoroughly with soap and water, using a sterilized brush. Then wash them in a solution of 1 to 1000 corrosive sublimate for 5 minutes, rinse thoroughly in boiled water, and wipe with a sterilized towel (furnished).

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 the bacteria. 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 XLII

DETERMINING THE THERMAL DEATH POINT OF BACTERIA

155. 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, in disinfection, and in pasteurization. For the various methods employed in making these determinations, see text-books and special articles on this subject. The method here given, 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. In this exercise students may work in groups with satisfactory results and with the saving of much media.

156. Work for this exercise. Take 10 tubes of bouillon; place 8 of them in a wire basket and stand it in the thermoregulated water bath at 60° C. After 15 minutes remove the tubes and inoculate 4 of them from a culture of *B. subtilis* and 4 of them from a culture of *B. suipestifer* or *B. coli* (the cultures will be furnished). In inoculating the tubes be sure not to touch the sides above the surface of the bouillon with the wire.

After the tubes are inoculated return them to the 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.

Inoculate the other two tubes of bouillon, one from each of the cultures used, and place them in the incubator for controls.

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 tubes to determine if they are pure.

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

Examine the cultures made from the scrapings of the skin in the previous exercise.

EXERCISE XLIII

DETERMINING THE EFFICIENCY OF DISINFECTANTS

157. 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.[1]

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

158. Work for this exercise. Put 10 cc. of a 2% solution of carbolic acid, prepared from sterile distilled water, into each of 2 sterile test tubes. Add to one of these tubes, by means of a sterilized pipette, .25 cc. of a bouillon culture of *B. coli* or *B. typhosus*. To the other tube add a like quantity of a suspension in bouillon or sterile water of an agar culture of *B. subtilis* (furnished).

Inoculate a tube of bouillon containing fully 7 cc. 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

[1] The student is referred to Bulletin No. 82. The Standardization of Disinfectants, Hygienic Laboratory, Public Health and Marine-Hospital Service. Washington, D.C. 1912.

to go to the bottom of the inoculated tube. Label each with the strength of the disinfectant and time of exposure and place it in the incubator. It should be noted that the adding of .25 cc. 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 of the disinfectant used and the time necessary 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.

Examine cultures that were heated at the previous exercise.

EXERCISE XLIV

PASTEURIZING AND STERILIZING MILK

159. 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 should be from 60° to 68° C. and the time for heating 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).

In this exercise students may work in small groups.

160. Work for this exercise. From the fresh milk provided, make 2 agar plates, using 1 and 2 loopfuls, respectively, of the milk. Put 25 cc. in each of 6 large test tubes and set one in the incubator and leave one at the room temperature. Boil two of them for 30 minutes in a closed water bath, and pasteurize the remaining 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 .25 cc. (measure with a graduated pipette) for the third. Place in the incubator

one of the tubes of milk treated by each process with the plate cultures, 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.

Examine the cultures made in Exercise XLIII.

EXERCISE XLV

EXAMINATION OF CERTAIN BACTERIA, FUNGI, AND PROTOZOA NOT STUDIED IN OTHER EXERCISES

161. 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 fungi and protozoa, such as the ray fungus of actinomycosis, the piroplasma of Texas fever in cattle, and the plasmodium of malaria in man, will be demonstrated. The trypanosomes of surra or dourine, which cannot be studied experimentally in the time allowed, may be examined microscopically from permanent preparations.

162. Work for this exercise. Examine and make drawings of bacteria, fungi, and protozoa demonstrated in the preparations furnished.

EXERCISE XLVI

AGGLUTINATION TEST FOR DIAGNOSIS

163. This test depends upon the fact that when the blood serum of an individual suffering with certain infections such as typhoid or glanders, or that has recently recovered from such infection, is added to a bouillon culture of the organism, the bacteria become less motile, in case of motile bacteria, 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 be used, i.e. those from 1 : 10 to 1 : 1000. The test has proved to be of much diagnostic value, especially in typhoid fever and glanders. For diagnosis of glanders the macroscopic method of determination is necessary. See Part II — The Diagnosis of Glanders.

On account of the accuracy of this reaction it is employed extensively in many laboratories for diagnosis.

164. Work for this exercise. Take one loopful of a fresh bouillon culture of typhoid bacilli (which will be furnished) and place it on a cover glass, add one loopful of diluted blood serum from a typhoid patient, or the blood of an immune guinea pig, and immediately make a hanging-drop preparation with a loopful of the mixture and examine. Note the effect on the 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 to 10 blood serum has been added.

Repeat the above test with the blood from animals affected with or immunized against *B. suipestifer*.

Examine a dried specimen of blood for this reaction. Add a few drops of bouillon or water 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.

Complete and hand in all laboratory notes.

165. Securing blood for the agglutination test. (*a*) *Preparation of dried blood.* Prick the finger or the lobe of the ear (if in 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.

(*b*) *Fresh blood.* Procure a drop of blood as in (*a*); add to it 10 drops of water on a glass slide or in a small test tube. Stir until the blood is dissolved. One loopful of this mixed with a similar quantity of the bouillon will give a dilution of 1 to 20.

(*c*) *Serum.* From a similar but deeper prick, or by drawing a few drops of blood from a vein with a hypodermic needle, secure a few drops of blood. Place them in the bottom of a small, short, sterile tube and allow the serum to ooze out. It is important to separate 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.

Blood serum may be obtained by filling a small (capillary) glass tube with the fresh blood, sealing it, and allowing it to stand until the serum collects on the top, when the tube may be broken and the serum drawn off with a fine-pointed pipette.

PART II

DIAGNOSIS

It is essential that one should appreciate the close relationship of the methods already studied to those that must be followed in practical diagnostic work. This means that in the diagnosis of various infections the practitioner must employ, for the determination or identification of the ætiological agents, methods like or similar to those used in laboratory instruction. Many of these methods can be actually employed in the field, while in other cases it is necessary to send the material to a laboratory for examination. When it is necessary to forward tissues to a laboratory it is essential that they should be sent in such a manner that when they reach the examiner they will be in a condition suitable for making the required examinations.

The infectious diseases which are commonly encountered in this latitude are largely of bacterial origin. It sometimes happens that diseases due to fungi and protozoa are met with. In certain cases a histological study of the tissues is necessary. In the following sections somewhat specific methods are given for the diagnosis of the more common infectious diseases encountered by the veterinarian.

I

SENDING TISSUES TO LABORATORIES FOR EXAMINATION AND DIAGNOSIS

It often happens that practitioners wish to send to a laboratory tissues from certain animals for the purpose of diagnosis. The diagnosis is made from the results obtained from one or more of the following examinations or tests; namely, the microscopic study of the tissues themselves, bacteriological examination, including animal inoculations, and specific reactions, such as the agglutination test. At certain times in the course of a few diseases it is possible for the experienced pathologist to tell from the gross lesions the nature of the affection. In order that the necessary examinations may be properly carried out and the diagnosis made, it is evident that the required tissues must be received in a suitable condition. The responsibility for obtaining a diagnosis from a laboratory falls very largely on the sender of the material. In selecting organs or tissues from a diseased animal for examination it is essential to choose parts containing the affected tissues. With infectious diseases this is especially important. In rabies, for instance, the necessary parts are in the brain and ganglia, in tuberculosis the lesion may be in any organ, while in anthrax, especially in cattle, the disease is general and any organ may be chosen. It is also necessary that a sufficient quantity of the tissues be sent.

Specimens should be accompanied by a letter stating what tissues were sent and what disease or diseases are suspected. This is necessary in order to facilitate the examination.

In sending tissues to a laboratory the following suggestions should be followed:

1. All tissues that are to be examined bacteriologically or in which the disease is to be diagnosed from the examination

of sections should be placed in tightly closed retainers which should in turn be well packed in ice. This should be done at all seasons of the year. The tissues should be sent as promptly as possible and by the quickest route.

2. In case of suspected rabies the entire head should be sent packed in ice. It need not be placed in a separate retainer. If the entire brain is removed and placed in glycerin, it can be shipped without ice.

3. Specimens for histological examination only or for museum purposes may be sent in 10% formalin.

II

ANIMAL INOCULATION FOR PURPOSES OF DIAGNOSIS

It is not always possible by the ordinary culture methods to determine the specific nature of a disease from a small piece of affected organ or tissue of the diseased animal. To make 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 others. Animal inoculation is further demandatory in determining the degree of virulence of pathogenic bacteria, or 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 the same, and in most cases are not the same, as those in the animal 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 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 would not interfere with the animal's locomotion.

III

THE DIAGNOSIS OF ACTINOMYCOSIS

Actinomycosis is caused by one of the higher bacteria belonging to the Streptothrices. It is commonly called the "ray fungus" because of its morphology. It occurs in the tissues in small granules which are stellately arranged threads projecting outwardly with the ends club-shaped. The positive diagnosis of actinomycosis consists, therefore, in finding the granules or ray fungus in the affected tissue or its discharges. In the case of purulent discharges the suspected material can be spread in a thin layer on a piece of glass or on a knife blade and examined with a low-power lens (hand lens) when the yellowish granules can be detected. In the laboratory the morphology of the ray fungus can be studied more accurately by placing the discharge on a slide, adding a few drops of 10% solution of caustic potash, covering with a cover glass, and examining with a moderately high-power objective.

In some cases the affected tissues are firm and must be sectioned and stained before making the microscopic examination. The stain commonly used for the ray fungus either in smears or in sections is carbol fuchsin. The Gram method is sometimes used. Cultures of actinomycosis are not usually employed in diagnosis.

If it is desired to send specimens of suspected actinomycosis to a laboratory for diagnosis, all or a portion of the affected part should be sent fresh and packed in ice.

IV

THE DIAGNOSIS OF ANTHRAX

The diagnosis of anthrax can be made from tissues of animals recently dead from that disease or from the living animal by the following methods:

1. Bacteriological examination of the tissues or blood.
2. M'Fadyean's stain of cover-glass preparations.
3. Ascoli's precipitation method.
4. Animal inoculation.

Bacteriological examination. The most favorable media for this organism is given in Exercise XXIII.

As there are many bacteria resembling the bacterium of anthrax[1] it is essential that its identification be based on a very careful study of the organism.

M'Fadyean's differential stain. M'Fadyean has described a peculiar staining reaction which he considers of value for the microscopic diagnosis of this disease. The reaction is in evidence when films of blood, exudates, or tissue juice containing the bacteria are stained with a simple aqueous solution of methylene blue. The method as applied to blood is as follows:

Place a drop of the blood on a clean slide. The size of the drop should be about 2 mm. in diameter. It is spread quickly with a platinum needle until it covers an area about 12 mm. in diameter. Protect from dust and allow the slide to remain until all evidence of moisture has disappeared. When dry, heat the preparation by lowering it, film upwards, into the flame of a Bunsen burner or an alcohol lamp for a second. Repeat

[1] The junior author summarized the characteristics of these organisms in the Report of the New York State Veterinary College, 1909–1910, p. 200. The essential difference between *Bact. anthracis* and the pseudo or anthraxlike organisms lies in the morphology (flagella) and pathogenesis.

this three times or until the glass is too hot to be borne by the skin in the palm of the hand. Allow the slide to cool and then cover the film with a 1% aqueous solution of methylene blue. After a few seconds pour off the free stain and wash the slide thoroughly in tap water. Dry the slide by pressing it gently between two layers of bibulous paper, and then more thoroughly by holding it in the current of hot air above the Bunsen flame. Finally, mount in Canada balsam.

The microscopic examinations (× 800 to 1000) will show an occasional leucocyte and the anthrax bacteria. There will appear no other visible formed elements. The nuclei of the corpuscles in general exhibit a greenish-blue tint, the anthrax rods are stained blue. The intensity of the stain depends upon the length of time after death before the films were made. Usually the segment character of all but the shortest rods will be apparent. If they are deeply stained this is not very distinct. *The peculiarity in the reaction lies in the color of the amorphous material which is present between and around the bacteria.* This material presents itself under the form of coarse or fine granules of a violet or reddish-purple color, which is in sharp contrast to the tint of the bacteria or cell nuclei, especially with brilliant lamp or gas light. These violet granules differ a good deal in form and size; sometimes they are very minute, at other times coarsely granular. When the bacteria are arranged in clumps the violet material is often in greatest amount about them. Free-lying anthrax rods will be surrounded by a thick envelope of the same substance. M'Fadyean states that he has never found this reaction in animals dead from other diseases. The peculiar coloring, he states, in some cases may be observed without the aid of the microscope.

Ascoli's thermo-precipitation method. This method is receiving considerable attention in Europe. It is based on the action of the specific antibodies in the blood and tissues of infected animals upon an immune serum. On account of the difficulty in obtaining the immune serum, this method is not

practicable except when large numbers of diagnoses are being made or when anthrax is suspected in material difficult to analyze bacteriologically.

See Annales de Médecine Vétérinaire, 60e année, N°. 6, p. 321, also Archiv. f. wiss. u. prakt. Tierheilk., 38 Bd. s. 207.

Animal inoculations. For this mice or guinea pigs are preferred. They can be inoculated either by placing small pieces of the suspected material under the skin or by crushing the tissue in sterile salt solution and injecting it hypodermically. Guinea pigs will die in from 1 to 3 days. Pure cultures may be obtained from the organs (liver, spleen, or blood). The bacteria appear in large numbers in cover-glass preparations or in sections of the liver, spleen, or kidneys.

When it is necessary to send specimens to a laboratory for diagnosis, the blood or any organ may be chosen when the disease is generalized, as is usual in ruminants. In animals where the disease is localized the specimen must be taken from the lesions. In case blood is sent, about 20 cc. should be drawn and securely packed. A diagnosis can often be made from the blood which is contained in the ear. Specimens should be taken immediately after the death of the animal.

It is important that blood of anthrax animals should not be allowed to contaminate the immediate surroundings.

V

THE DIAGNOSIS OF BLACK LEG

The diagnosis of black leg (symptomatic anthrax) is made from specimens of the lesions sent to a laboratory by isolating the specific organism (*B. Chauveaui*) either by cultures or by guinea-pig inoculations. Cultures should be made anaërobically, preferably in glucose agar (Liborius' Method) or in glucose bouillon in the fermentation tube and incubated at about 37° C. Growth appears in from 24 to 48 hours, when the cultures should be examined.

The most accurate means of diagnosing black leg is by guinea-pig inoculation. Portions of the affected tissue should be placed beneath the skin of the pig, or the tissue may be ground in a mortar with sterile bouillon and the suspension injected subcutaneously with the hypodermic syringe. If the material contains virulent black-leg organisms the pig will die in from 1 to 3 days. It should then be examined for the lesions of black leg, such as subcutaneous œdema and emphysema. A microscopic examination of smears made from the diseased parts of the guinea pig will reveal the presence of the organisms.

Cover-glass preparations made from the fresh lesions of the affected animal will usually reveal the presence of the specific organism. In order to be sure of its identification it is necessary to resort to cultures or guinea-pig inoculations or both. It should be remembered that usually the bacilli of black leg are not present in the general circulation or in the internal organs. In certain cases, however, it can be recovered from the spleen or liver.

If laboratory aid is required to make the diagnosis it is necessary to send a portion of the affected muscle. It is also desirable to send the spleen.

VI

THE DIAGNOSIS OF GLANDERS

The laboratory diagnosis of glanders consists of several methods, one or more of which may be used according to the conditions. If the material is from a dead animal the methods are restricted to bacteriological and histological examinations of the tissues. If the material (nasal discharge or pus from abscess on skin) comes from the living animal there is the possibility of making not only a bacteriological examination of this material but also of having blood taken from the horse for applying the two serum tests now recognized to be of value. The methods for diagnosing glanders, therefore, are

1. Bacteriological (cultures).
2. Guinea-pig inoculation.
3. Agglutination test.
4. Complement-fixation test.
5. In the living horse mallein may be applied.

The bacteriological method is described in Exercise XXVI.

Animal inoculations. Male guinea pigs should be used. The material usually consists of the nasal discharge from the suspected glandered horse, or bits of scrapings from ulcers, or pieces of affected tissue. The inoculation is liable to be followed by a local swelling and an abscess. The first indication of glanders 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 from the suppurating focus of the testicle. The spleen is usually enlarged and sprinkled with grayish nodules. Other organs may be involved.

Agglutination test. The method is as follows:

Culture. As pointed out by Schütz and Miessner all cultures of *Bact. mallei* do not agglutinate satisfactorily. It was also

shown by their work that a suitable culture, when obtained, is liable, at unexpected intervals, to lose its responsiveness to the agglutinin. This can often be prevented by passing the organisms through a guinea pig. The organisms should be grown for from 48 to 72 hours on acid glycerin agar (5 % glycerin with a reaction of + 2.9 to phenolphthalein). In order to have a suitable culture on hand, subcultures should be made at short intervals. A culture about 72 hours old should be used in preparing the test fluid.

Test fluid. The test fluid is prepared by washing the growth from the agar culture by the aid of a sterile wire loop into distilled water containing 0.85 % sodium chloride and 0.5 % carbolic-acid crystals. This suspension is then placed in a thermostat at 60° C. for 2 hours, which kills the bacteria. A temperature higher than 65° C. or lower than 60° C. should be avoided. After heating, the suspension is thoroughly triturated and filtered through sterile cotton. Thorough trituration of the emulsified growth is essential before filtering. The filtrate thus prepared is diluted with the carbolized salt solution until it is of a faintly clouded appearance. The proper dilution of the filtrate can only be determined by experience. The test fluid gives the best results when made with freshly prepared carbolized salt solutions.

Procuring the serum. The serum is easily obtained. At least 10 cc. of blood should be drawn from the jugular vein, under aseptic precautions, into a small sterile bottle, and sent to the laboratory. As soon as the clot forms, the supernatant serum is placed in a centrifuge and all the sediment thrown down, leaving the liquid perfectly clear. One cc. of the serum is then added to 39 cc. of a physiological salt solution, which makes a dilution of 1 to 40. It is desirable that the serum should be secured as soon as possible after the blood is drawn. If necessary to delay the test, the serum has given the best results if kept at about 10° C. until used.

Making the test. Three cc. of the test fluid are placed in each of several small test tubes. With a sterile pipette, the diluted serum is added to the tubes of test fluid and thoroughly mixed. In making the different dilutions, the amount of diluted serum to be used is readily ascertained by the following table:

Dilution of Serum	Amount of Diluted Serum	Amount of Test Fluid	Dilution
1–40	1.2 cc.	3 cc.	1–100
1–40	0.6	3	1–200
1–40	0.405	3	1–300
1–40	0.3	3	1–400
1–40	0.24	3	1–500
1–40	0.195	3	1–600
1–40	0.15	3	1–800
1–40	0.12	3	1–1000
1–40	0.105	3	1–1200
1–40	0.09	3	1–1500
1–40	0.06	3	1–2000
1–40	0.03	3	1–4000
1–40	0.015	3	1–8000

Where dilutions greater than 1 to 1000 are made, a serum diluted 1 to 80 may be used to better advantage, unless the pipette employed is very finely graduated. In this case the amount of diluted serum for a certain dilution must be double that indicated in the table.

The mixture thus prepared is placed in an incubator at 37° C. for 24 hours. A temperature higher than 37° C. interferes with the agglutination.

Reaction. The reaction consists of a layer of the agglutinated bacteria settling and covering the entire convexity at the bottom of the tube. This filmlike sediment may become so dense that it rolls in at the periphery. The supernatant fluid becomes clear in the lower dilutions, but in the higher ones the clarification may not be complete, showing that all the bacteria have not become agglutinated. This is further

evinced by the fact that the layer is less dense in the higher dilutions. The reaction may begin in 6 hours, but cannot be considered complete until from 24 to 72 hours have elapsed. Often, however, a reaction appears in less than 24 hours.

After the agglutination is completed, further standing produces no visible change in the test fluid.

A negative result shows a small round concentrated spot of sediment in the center of the convexity at the bottom of the tube, the test fluid remaining apparently unchanged even after several weeks.

An agglutination in a dilution of 1 to 500 is considered suspicious.

An agglutination in a dilution of 1 to 800 is very suspicious.

An agglutination in a dilution of 1 to 1000 is considered a positive reaction.

Complement-fixation method. This method is one that requires considerable special equipment and material, such as a powerful centrifuge, immunized rabbits and sheep for corpuscles. The method is considered to be very accurate. Those who wish to use it are referred to Bulletin No. 136, Bureau of Animal Industry, and to Moore's *Principles of Microbiology* for technique.

If laboratory aid is required the blood for the serum tests should be drawn aseptically in sterile bottles. About 20 cc. of blood is sufficient. Securely pack and send. In case of organs the parts containing the lesions should be sent.

VII

DIAGNOSIS OF HOG CHOLERA

Hog cholera is the epizoötic disease of swine caused by a filterable virus. The virus is not pathogenic for the small experimental animals, and as yet it cannot be cultivated. This restricts the means of diagnosis to the nature of the lesions and the inoculation of swine with the blood of the hog under suspicion. As this inoculation is expensive, and as it usually requires from 8 to 12 days for results, the diagnosis is to be made from the nature of the lesions by the practitioner in the field. It is often necessary to examine several hogs before one showing characteristic lesions will be found.

Lesions. The lesions by which hog cholera is diagnosed are petechial hemorrhages, especially in the kidneys, lungs, or in the heart muscle. Sometimes they are very numerous and pronounced; in other cases they are few and very small. Button ulcers in the intestine are sometimes present. Congestion and perhaps hemorrhage in the bone marrow are significant lesions. Congested and hemorrhagic lymph glands are suggestive. There is often reddening of the skin over the ventral surface of the body and about the head. The petechial hemorrhages are, however, the most characteristic lesions.

Inoculation. The diagnosis by inoculation consists in filtering the blood of the suspected hog through a Berkefeld filter, and inoculating a healthy pig subcutaneously with about 2 cc. of the filtrate. It will develop symptoms of hog cholera in from 5 to 8 days and will die in from 8 to 15 days if the blood came from a pig suffering with hog cholera.

Where hog cholera is suspected it is necessary to send the entire viscera. It is often desirable to send the pig itself. It is absolutely necessary that the tissues arrive in a fresh condition.

VIII

DIAGNOSIS OF RABIES

The laboratory diagnosis of rabies is restricted, for all practical purposes, to three procedures; namely,[1]

1. Inoculation of experimental animals with the brain of the suspected case.

2. The determination of the changes in the ganglia as described by Van Gehuchten and Nelis.

3. The presence of Negri bodies.

(1) In the animal inoculation method rabbits are to be preferred, although guinea pigs may be used. The animals should be inoculated with the suspected brain, preferably subdurally, although intraocular and intramuscular inoculation can not be entirely excluded. The diagnosis by this method is not recommended, except in (*a*) case of failure by the other methods, and (*b*) where a late determination will be of value.

The subdural method of inoculation 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 centimeters 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,

[1] Report of Committee on Standard Methods for the Diagnosis of Rabies. Read before the Laboratory Section of the American Public Health Association, Milwaukee, September, 1910.

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 in 15 days, and in some cases the rabbits are not attacked for from 1 to 3 months.

The symptoms following the inoculations have in our 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 movement. 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°, which continued for a variable length of time, but rarely longer than 2 days. This is followed by a gradual but usually rather rapid drop to the subnormal, which continues to the end.

(2) The diagnosis by means of the changes in the Gasserian ganglia, described by Van Gehuchten and Nelis, has proved to be very satisfactory where the suspected animal has died or was killed in the late stages of the disease, or when Negri bodies cannot be found or cannot be looked for because of putrefaction, the destruction of the brain, etc. When sections are made from the ganglia removed during the first stages of the disease, the apparently specific lesions are often absent or not sufficiently well marked to warrant a diagnosis. The changes may be of two kinds; namely:

The typical, or focal, lesions. These are characterized by the partial or complete destruction of the ganglion cells, their place being occupied by cells of the endothelial type. The foci may consist almost entirely of cells of the endothelial type, but often associated with them are mast cells, lymphocytes, plasma, connective-tissue cells, and polymorphonuclear leucocytes.

The atypical, or diffuse, lesions. These are characterized by a more or less general infiltration between the ganglion cells and nerve fibers of cells of an endothelial origin and the various cells which are associated with chronic inflammatory processes. These changes are usually associated with the typical lesions.

The lesions in the ganglia may be very extensive, involving the entire ganglion, or they may be restricted to a single ganglion cell here and there in the organ. The lesions may be present in one ganglion and not in another. It is sometimes necessary to examine a number of sections before finding the changes.

(3) The diagnosis by the presence of Negri bodies. These bodies may be found in a microscopic examination of properly stained preparations made in either one of three ways; namely, (*a*) Sections of the affected brain properly fixed and stained. (*b*) Impression preparations of the Ammon's horn or cerebellum. (*c*) Smears.

The technic of the smear method is as follows:

(1) A small bit of the gray substance of the brain chosen for examination is cut off with a sharp pair of scissors and placed about one inch from the end of a glass slide so as to leave room for a label. The cut in the brain should be made at right angles to the surface.

(2) Another glass slide is placed over the piece of tissue and pressed upon it until it is spread out in a moderately thin layer, then this latter slide is moved slowly and evenly across to the end opposite the label. Only slight pressure should be used in making the smear, but slightly more should be exerted on the slide toward the label end, thus allowing more of the nerve tissue to be carried further down the smear and producing more well-spread nerve cells. If any thick places are left at the edge of the smear they should be spread out toward the side of the slide.

(3) Such a smear should be made from at least three different parts of the brain. First, from the Ammon's horn; second, from the cerebellum; third, from the cortex in the region of the crucial fissure.

(4) These smears are fixed in heat, preferably over a mild flame from a Bunsen burner. Care must be taken not to heat too much.

(5) The smears are then stained for from 10 to 15 minutes in a saturated solution of alcoholic eosin. This stain works better if it is at least 3 months old.

(6) Remove smear and wash in tap water. Dry carefully between filter paper and stain for from 10 to 30 seconds with Loeffler's alkaline methylene blue.

(7) Wash in tap water, dry, and dehydrate for from 5 to 20 seconds in absolute alcohol.

(8) Clear in xylene.

(9) Mount in balsam.[1]

The Negri bodies appear as round or oval purplish-red bodies lying outside of the nucleus within the protoplasm of the large nerve cells.

The following procedure is recommended in diagnosing rabies from suspected animals sent to laboratories for this purpose :

1. Examine for Negri bodies, and if they are found, report the disease as positive.

2. If Negri bodies cannot be found, examine the Gasserian ganglia for cellular lesions.

3. If the results from the examination for Negri bodies and ganglion changes are negative, rabbits should be inoculated in those cases where a late diagnosis will be of value. Otherwise, animal inoculations should be omitted.

In suspected rabies the entire head of the animal should be packed in ice and shipped. However, the entire brain may be removed and placed in glycerin. It is very desirable that in the latter case the brain be removed without laceration.

[1] The smear may be examined without balsam and a cover glass.

IX

DIAGNOSIS OF SWINE PLAGUE

Swine plague is an infectious pneumonia of swine caused by *Bact. suisepticus*. The lesions are usually restricted to the lungs. The diagnosis is made from a bacteriological examination of the lungs. See Exercise XXIX for cultures. A very satisfactory method is to inoculate rabbits subcutaneously with small pieces of the affected lung. They will die in from 18 hours to a few days, according to the virulence of the organism. From the heart blood, spleen, or liver pure cultures of the organism can be obtained.

When sending to a laboratory for the diagnosis of swine plague the entire lungs and heart of the pig should be forwarded. As hog cholera is often associated with swine plague, it is desirable to send in addition to the lungs and heart the kidneys and such portions of the intestines as show any abnormal condition.

X

THE DIAGNOSIS OF TUBERCULOSIS

The laboratory methods of diagnosing tuberculosis consist in finding the tubercle bacteria in the suspected material. In the living animal, tuberculin can be applied to determine whether or not a reaction will occur. With suspected tissues in the laboratory the following methods may be used:

1. Bacteriological methods.
2. Animal inoculation.

The methods of staining tubercle bacteria and media for their cultivation were described in Exercises XVII and XXVIII.

Cultivation of tubercle bacteria. To isolate this organism from tuberculous lesions and cause it to multiply readily on artificial media necessitates a special and very careful procedure. When it becomes accustomed to artificial media its continued cultivation is not difficult. Dr. Theobald Smith of Harvard University (*Journal of Experimental Medicine*, 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:

"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 tubercules. 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, molds 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 molds 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.

Animal inoculation. The guinea pig is preferable for diagnosis. With tuberculous tissues either of two methods may be employed: (1) a piece of the tissue about the size of a pea or bean may be inserted under the skin by first making an incision through the skin and superficial fascia with a sharp scalpel and then with a pair of fine forceps, inserting the bit of tissue well under the skin and closing the opening with one or more sutures; (2) The tissue may be crushed in a mortar and thoroughly mixed with a few cubic centimeters of sterile water or bouillon and then injected with a hypodermic syringe. The needle should be of large caliber. If suspected milk is being examined 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 3 weeks to 4 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.)

If it is desired to differentiate between the human and the bovine varieties it is necessary to use rabbits, as the bovine variety is generally pathogenic for rabbits and the human variety rarely, if ever, is.

In case tuberculosis is suspected, all or, if too large, a portion of the lesion should be sent.

XI

THE DIAGNOSIS OF PROTOZOAN DISEASES

There are two diseases caused by trypanosomes and one by piroplasma that are liable to be encountered in this country; namely, dourine, surra, and Texas fever. Surra has never been found in this country, but it has been brought to our quarantine station, and it is possible that some carrier of the parasite may introduce it. Dourine, however, has occurred in several places in the United States and Canada, and therefore may be met at any time. Texas fever prevails in certain sections of the South. It is occasionally brought into the Northern states.

Surra. In cases of this disease the trypanosome may be found by a microscopic examination of the blood, either in the fresh condition or in stained cover-glass preparations when taken at the proper time during the active course of the disease. They are present in the peripheral blood for a short time only and then disappear. In case of suspected surra one can inoculate subcutaneously mice, rabbits, or dogs with a few cubic centimeters of the blood and find the parasites in their blood after the symptoms appear or at death. The parasites are reported to be scarce in the rabbit's blood but numerous in that of mice.

Dourine. The trypanosome of dourine is not usually found in the blood of the infected horse. It is, however, present in the œdematous swellings caused by it and in the serum in the early stages of the plaques which occur in many cases. The diagnosis of dourine by the presence of the parasite can be determined, as a rule, only from a study of them in the local lesions.

Texas fever. In fatal cases of Texas fever, one can make smear preparations from the liver, kidney, or heart muscle. The *Piroplasma bigeminum* are more numerous in the capillaries.

Films are made from the organs or blood and fixed in the usual manner. They are stained with alkaline methylene blue the same as those of bacteria.

Staining blood films for animal parasitea (protozoa). Several methods of staining blood films to demonstrate blood parasites are in use. Alkaline methylene blue stains most, if not all, parasitic protozoa, but the following (Nocht-Romanowsky stain) gives very satisfactory results.

Preparation of the stain. To 1 ounce of polychrome methylene blue (Grübler) add a 3% solution of acetic acid (U.S.P., 33%), drop by drop, until it no longer turns red litmus paper blue above the zone coming into immediate contact with the dye. It usually requires about 5 drops.

2. Make a saturated (1%) watery solution of methylene blue, preferably Ehrlich's (Grübler) or Koch's, dissolving the dye by gentle heat. This solution improves with age and should be at least 1 week old.

3. Make a 1% watery solution of Grübler's watery eosin.

The mixture is prepared as follows:

To 10 cc. of water add 4 drops of eosin solution, 6 drops of neutralized polychrome blue, and 2 drops of 1% methylene blue, mixing well.

Wright's stain is excellent for this work. It can be bought in the market. This is also true of Jenner's stain.

Preparation of films for the staining of protozoa in the blood. The blood should be drawn from the skin rather than from the larger veins. It is obtained by shaving the surface, cleansing it, and making a small incision with a scalpel or lance. Apply the edge of one end of a glass slide to a small drop of blood and place this edge on the surface of another slide resting on a firm surface. As soon as the drop of blood has spread along the line of contact, holding the slide at an angle of about 30°, draw it gently along the surface of the receiving slide, spreading the blood in a thin film. Allow the films to dry in the air before fixing.

Fixing the blood films. 1. By heat. (*a*) *Open flame.* Pass the slide, specimen-side up, slowly through the flame of a Bunsen burner until it is decidedly too hot for the hand to bear. At this temperature, which probably varies from 110° to 150° C., fixation is complete in from 1 to 2 minutes. Overheated slides can usually be seen to change color in the flame, after which the red cells stain yellow with eosin. A little practice will enable one to tell when fixation is complete without overheating. (*b*) *Ovens.* Slides may be placed in an oven provided with a thermometer and exposed to a temperature of from 110° to 150° C. from 5 to 10 minutes.

2. By alcohol. Fix in from 95% to 97% alcohol from 10 to 30 minutes. If left too long in alcohol they do not stain so well. For staining malarial parasites, fixation by alcohol is preferable.

Method of using Nocht-Romanowsky's stain. Put the stain in a Petri dish, place in it two or three toothpicks, matches with heads removed, or pieces of small glass rod to support the slides, and place them, specimen-side down, in the stain upon this support. This allows any precipitate to settle away from rather than upon the slides. Allow the stain to act 1 or 2 hours. They will not overstain in 24 hours. Wash in water, and when dry they are ready to examine without a cover glass. Immersion oil can be applied directly to the films without injury.

XII

DIAGNOSIS OF NON-SPECIFIC LESIONS

There are many diseased conditions found in tissues, such as tumors, inflammatory processes and degenerations, that require for an accurate diagnosis a careful histological examination.

In sending tissues for this purpose the part to be examined can be put immediately in 10% formalin or in alcohol. Usually, however, it is better to send the lesion in a fresh condition so that the examiner can select the portions to be sectioned in order to ascertain the true nature of the morbid process.

APPENDIX

I

REACTION OF CULTURE MEDIA

THE importance of the reaction of media as a controlling factor in the development of biological characters is such 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 dioxid 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 dioxid.

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."

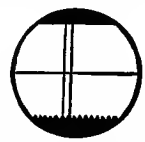
Journal Am. Public Health Association, January, 1898.

II

THE OCULAR MICROMETER AND MICROMETRY[1]

"**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.



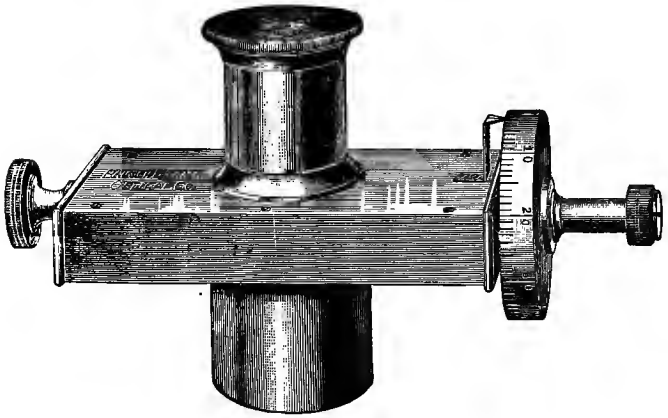
Field of large filar micrometer showing cross hairs and recording comb.

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).

[1] These 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.

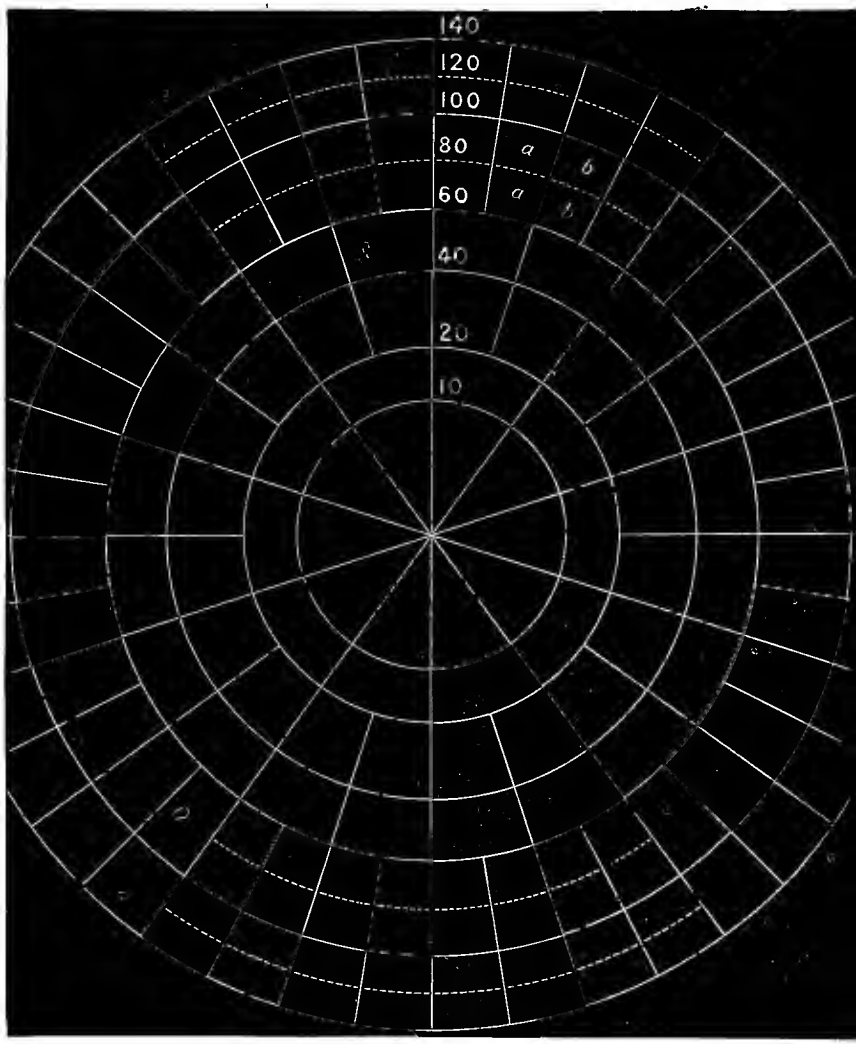
Divide the value of the included space or spaces on the stage micrometer by the number of divisions on the ocular micrometer required to include them, and the quotient so obtained will give the valuation of the ocular micrometer in fractions of the unit of measure of the stage micrometer. For example, 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. $10=\frac{1}{100}$ 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. JEFFERS PLATE

For Counting Colonies of Bacteria in Petri Dishes.

The area of each division is one square centimeter.



IV

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), 1/25000th 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. × 9/5) + 32 = F. For example, to find the equivalent of 10° Centigrade, C. = 10° (10° × 9/5) + 32 = 50° F.

To change Fahrenheit to Centigrade: (F. − 32°) × 5/9 = C. For example, to reduce 50° Fahrenheit to Centigrade, F. = 50°, and (50° − 32°) × 5/9 = 10° C.; or − 40 Fahrenheit to Centigrade, F. = − 40° (− 40° − 32°) = − 72°, whence − 72° × 5/9 = −40° C.

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

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