COLUMBIA UNIVERSITY.
COLLEGE OF PHYSICIANS AND SURGEONS.
DEPARTMENT OF BACTERIOLOGY.

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Laboratory Manual
in Medical Bacteriology

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Laboratory Manual **IN** **Medical Bacteriology**

**Edited by the
Staff of the Department of Bacteriology**

**College of Physicians and Surgeons
Columbia University
New York**

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LABORATORY MANUAL IN MEDICAL BACTERIOLOGY

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Staff of the Department of Bacteriology

Third Edition

College of Physicians and Surgeons
Columbia University
New York

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INTRODUCTION

Bacteriology as a general science is a subdivision of Botany. Bacteria may be studied taxonomically as plant forms, or practically in their relation to various industrial processes. The overwhelming importance of certain bacteria as the agents of diseases in plants, animals, and man have set aside the pathogenic bacteria as a specialized group around which has been created one of the most important branches of medicine. Medical bacteriology, viewed from another angle, is the logical introduction to Pathology which underlies the whole practice of medicine.

Pathology is the science of disease. In its larger sense the term may be used to designate a group of closely correlated medical sciences and also the applied aspects of these sciences in the recognition and treatment of disease. The function of instruction in Pathology is to serve as an introduction to the study of clinical medicine, to outline the abstract conceptions of disease processes, to point out the variations from normal function and structure which occur in the animal body as the result of disease.

The complete consideration of any particular disease would logically consider, first, the cause of the disease, secondly, its course, and thirdly, its effect on the normal body. We find that diseases are due to three general causes: (1) external inanimate agents, (2) external animate agents, and (3) a set of causes as yet unknown. The diseases due to external animate agents, or the infectious diseases, as they are called, comprise about one-half of all disease entities, and are the cause of approximately one-half of the deaths. The infectious diseases, therefore, are numerically important and possess the additional advantage of affording facilities for study in their three successive phases, as already outlined. The microorganismal causes of disease can be, for the most part, isolated outside the animal body and studied separately (bacteriology and protozoology). Their effects can be watched in the animal body and the changes induced therein studied (infection and immunity) and finally the resultant effects or lesions of the diseases they produce can be examined (morbid anatomy and histopathology). In the diseases due to external inanimate agents

and in those due to unknown causes, the origin in specific instances is not always recognizable and the sequence of events is frequently obscured. The final results as measured by metabolic and structural changes are, however, rather more striking than in the infectious diseases. Many of the effects produced can be simulated by the methods of experimental pathology. These latter studies in the experimental animal serve, moreover, as an introduction to the more extensive investigations of diseased function and structure that will be taken up in hospital work.

This syllabus outlines a laboratory course in:

(1) The methods of general bacteriology. The principles of asepsis, sterilization, preparation of culture media. The isolation in pure culture and identification of various saprophytic bacteria with some conception as to the ubiquity of these micro-organisms in life. The biochemistry of bacterial growth.

(2) An intensive study of the commoner and more important bacteria that cause disease in man and animals.

(3) The reproduction experimentally in animals of certain infectious diseases by inoculation of their respective bacterial agents (Infection).

(4) A study of the defense mechanism in the animal body against bacteria (Immunity).

The laboratory work is presented in the form (1) of exercises which state the more important technical details and the well recognized facts which should be studied by all those who learn bacteriology and (2) problems of a more difficult and experimental nature to be assigned to and worked on by individual students who will present their results to the whole class.

References to current literature are given in footnotes; a consultation of some of them is recommended, in addition to textbook reading, in connection with the class exercises, and will be essential in order to understand and perform the individual problems.

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Zeitschrift für Hygiene und Infektionskrankheiten	Berlin.
Zeitschrift für Immunitätsforschung und experimentale Therapie; Originale, Referate	Jena.

GENERAL INSTRUCTIONS

Desk and Locker Space:

Each student should be assigned a desk with locker, a separate locker for tray of stains and reagents, and a tray in the incubator room.

Supplies and Apparatus:

Students will be supplied with a tray of stains and reagents, Bunsen burner and tubing, ring stand, granite cup and thermometer. Each student should provide the following individual equipment:

- One textbook.
- One microscope.
- One apron, waiter's coat or laboratory smock.
- Two towels.
- One handkerchief for cleaning slides
- One set of dissecting instruments.
- One pair of slide forceps.
- Two platinum or nichrome needles with holder.
- Two rubber nipples.
- One box of microscope slides.
- One box cover slips, No. 1, 22mm, (circles or squares.)
- One package of lens paper.
- 2 padlocks and keys for lockers.
- One laboratory notebook.
- One wax pencil (glass or skin-marking).
- One small triangular file.
- 4 hollow-ground slides.

General Rules:

The wire used in making cultures should be sterilized by heating to redness in the Bunsen flame before and after every use made of it. If it is covered with viscous material, dry at the side of the flame before sterilizing to avoid scattering infectious material.

Sterilize hollow-ground slides and slide cover-slip preparations by placing in the jar of disinfectant provided. Displace the cover-slip with forceps or needle, which should then be flamed.

Instruments that have been used on infected animals should be boiled before putting away.

Cultures and infected tubes that are to be discarded should be left on the desk; they will be collected and sterilized after the class session.

Put non-infectious solid waste into the buckets provided. Turn Bunsen burners low when not in use, to avoid over-heating the laboratory and wasting the gas. Watch for gas leaks. At the close of the day's work, put all cultures to be studied further, in the incubator, or when directed, in the desk locker; put away all apparatus, and wash the hands with soap and water.

Accidents:

In case a living culture is spilled accidentally, notify one of the instructors and cover immediately with disinfectant. If the fingers come in contact with living culture material, rinse the hands in disinfectant and scrub with soap and water. In case of personal accidents, such as cutting or pricking the fingers, or splashing infective material or culture in the eye, report at once to the instructor in charge. Use every precaution to avoid such accidents. Smoking is not permitted during class hours. Keep fingers and objects in use away from the mouth.

Use and Care of the Microscope:

Good illumination is essential in the examination of bacteria. Use the plane mirror for daylight and the concave mirror when using electric light for illumination. The sub-stage condenser should always be employed; it should be raised or lowered slightly with slides of varying thickness to focus the rays of light sharply upon the field to be examined. The usual Abbé Condenser is satisfactory but the aplanatic condenser gives more brilliant illumination.

When the low-power objective is used the iris diaphragm should be closed to a very small diameter; with the high-power dry objective it should be opened slightly and with the oil immersion lens it should always be wide open.

Oil immersion objectives containing a fluorite system yield the most perfect definition. In the use of the oil immersion lens, place a drop of cedar oil on the portion of the slide which is to be examined and lower the objective with the coarse adjustment until the front lens touches the drop of oil. Observe the lens from the side and continue to lower the lens until it almost touches the slide. Then apply the eye to the ocular and focus up slowly with the coarse adjustment until the field comes into view. Subsequent focusing is done with the fine adjustment.

Before putting the microscope away carefully wipe off the cedar oil from the immersion objective with lens paper, using xylol to soften and remove gummed oil.

EXERCISE 1

Handling of Bacterial Cultures

Bacteria are cultivated most commonly in tubes, Petri plates, and flasks, which are used to contain liquid media such as nutrient broth, and similar media which have been brought to a semi-solid or jelly-like consistency by the addition of agar-agar or other substances. The composition of the media commonly used and their methods of preparation are given in a separate section in the back of the manual.

In opening tube and flask cultures, the cotton stopper is removed with a rotary motion, while the tube or flask is held inclined toward the horizontal plane; the mouth of the tube or flask is then passed through the Bunsen flame to destroy bacteria which cling to shreds of cotton or particles of dust that might fall into the culture.

Samplings of the cultures are made with a wire fixed in a holder. The wire should be of nichrome or platinum, of 26 to 28 B. & S. gauge, and about 7 cm. in length. It may be used in a special holder or may be welded in a very hot Bunsen flame or blast-lamp to a rod of aluminum or glass 3 to 5 mm. in diameter. Before and after every use of the wire it should be sterilized by holding in the Bunsen flame until it is heated to redness. One wire should be straight, the other should have a loop 2 mm. in diameter made in the free end by means of round-nosed pliers.

After the culture is sampled the mouth of the tube should be flamed again and the stopper returned. While the stopper is out of the tube it should not be allowed to come into contact with any object.

The micro Bunsen burner held by a burette clamp in the horizontal plane gives the most satisfactory flame but is not generally employed.

Semi-solid or solid media only are used in Petri plates. Cultures in Petri plates are protected against contamination by the cover. In sampling, the cover should be raised just enough to permit the wire to be used freely. Petri plate cultures are inverted before being stored or placed in the incubator, in order to prevent the liquid expressed from the medium, the so-called water of condensation, from flowing over the medium and carrying contamination from the edges of the plate.

EXERCISE 2

Fresh Preparations

Material needed:

24 hour broth cultures of
Staphylococcus albus
Streptococcus viridans
Bacillus pyocyaneus
Bacillus subtilis

The hanging drop is often used in the examination of fresh preparations. Spread a thin ring of vaseline with a tooth-pick about the concavity of a hollow ground slide. With the flamed loop, place a drop of the fluid to be examined in the center of a clean cover slip resting on a flat place on the desk. Then invert the hollow ground slide over the cover slip and press gently down to make the ring of vaseline adhere to the cover slip, with the drop in the center of the concavity. Invert the slide again and it is ready for examination.

A slide cover-slip preparation answers almost every purpose of the hanging drop, and has the advantage of distributing the bacteria more nearly in a single plane. This facilitates focussing and there is less danger of crushing the cover slip with the lens. Such a preparation is made by placing with the flamed loop a small drop of the fluid to be examined on a slide, and placing a cover slip over the drop so that it spreads out in a thin film between cover slip and slide.

Examination of either preparation should be made first with the low, than the high power dry lens, which usually gives sufficient magnification. In exceptional cases the oil-immersion lens is used; it is especially valuable with dark-field illumination in examination of the spiral organisms.

Make a fresh preparation from each of the bouillon cultures. Observe the morphology of each organism. Examine for motility: distinguish between (1) true motility, shown by movement across the field of vision, (2) Brownian motion, which is a rapid vibratory or dancing movement due to molecular forces, and (3) uniform movement of many individuals due to currents in the fluid in which they are suspended.

EXERCISE 3

Stained Preparations

Material needed:

- Agar cultures of *Staphylococcus albus*
- Bacillus coli*
- Bacillus subtilis*
- Killed suspension of *Bacillus tuberculosis*

While *en masse* some bacteria are pigmented, microscopically the individuals are nearly colorless. Their morphology is therefore best studied in stained preparations. There are many stains for special purposes; only the principal bacterial stains are mentioned here. They are best demonstrated with 24 hour cultures on plain agar. Staining solutions are supplied to students ready to use. The formulas are to be found on page 178.

Preparation of Films:

Take a clean glass slide and mark and number three divisions with a wax pencil. With sterilized loop, place a very small drop of water in the center of each division. The slide must be clean and free from greasy material, as otherwise the water will not adhere to the glass and it will be impossible to spread the drop out. Flame the loop. Open a culture tube and flame its mouth, barely touch the culture growth with the loop, and emulsify the material taken up in one of the drops of water on the slide. Flame the mouth of the tube and replace the plug after withdrawing the loop. Spread the drop in a thin film over an area of about one square cm. Make similar films of other organisms in the other drops.

Allow the film to dry spontaneously, or in damp weather accelerate drying by holding the slide high over the flame at a temperature which does not burn the hand. Fix the film, when thoroughly dry, by passing the slide three times through the flame, with the film side uppermost. Cool before staining.

1. Simple stain.

Cover the fixed film with Loeffler's methylene blue. Allow to stand 1 minute. Wash, drain, and blot; dry thoroughly by holding high above flame. Safranin or dilute carbol fuchsin may be used in a similar way.

2. Gram stain.

A. Cover fixed film with Sterling's gentian violet, 15 sec.

B. Wash off gentian violet with water.

C. Cover with Gram's iodine solution and allow to stand 15 seconds or until the film has assumed throughout a grayish brown color.

- D. Wash off iodine with 95% alcohol, changing alcohol two times, and allow final alcohol to remain on slide 1 minute.
 - E. Wash with water.
 - F. Cover with safranin one minute. Bismarck brown two minutes or dilute fuchsin $\frac{1}{2}$ minute, also make good counter stains.
 - G. Wash with water, blot and dry.
3. Ziehl-Neelson stain (acid-fast stain).
- A. Prepare film from suspension of *B. tuberculosis*, and on another portion of the same slide one of *B. subtilis*. Fix by heat, avoiding overheating.
 - B. Place slide on ring stand, cover slide with carbol fuchsin and heat with Bunsen flame until the stain begins to steam. Heat occasionally, and keep the slide steaming for three minutes. Do not let the stain boil; add more stain if necessary before the film dries.
 - C. Wash with water.
 - D. Decolorize with several changes of acid alcohol, until no more dye is removed.
 - E. Wash with water.
 - F. Counterstain with Loeffler's methylene blue one minute.
 - G. Wash, blot and dry.

The student should make stained preparations from each of the agar cultures, using simple and Gram stains, and an acid fast stain of *B. tuberculosis* and another organism. Examine the stained films with the oil immersion lens. In the methylene blue stains, look for metachromatic granules or areas of irregular staining in the bacteria. With the Gram stain, observe the difference in final color. Organisms retaining the gentian violet color are spoken of as Gram positive while those which lose the purple color and take the counterstain are called Gram negative. The technic of the Gram stain should be repeated until the student is certain of his results.

In the acid-fast stain, if the staining has been properly carried out, the background takes the counterstain, and only certain organisms that possess a waxy material preserve the red color of the carbol fuchsin after the treatment with acid alcohol. Such organisms are called acid-resistant, or acid-fast.

Make a careful study of each organism with the different stains. Sketch and write brief description in note-book.

EXERCISE 4

Inoculation of Culture Media

A. Material needed:

- 24 hr. broth culture of staphylococcus albus
- 1 plain agar slant
- 1 bouillon tube
- 1 gelatin tube
- 2 deep agar tubes
- 1 Petri plate

1. The technic of inoculation of different culture media will be demonstrated by the instructor. Students will make inoculations, from stock cultures supplied, upon:
 - (a) Plain agar slant
 - (b) Bouillon tube
 - (c) Gelatin tube (Stab culture)
 2. Place two deep agar tubes in agate cup filled with water and heat to boiling; at this temperature the agar melts. Cool water to 45°C.
 3. Inoculate one tube of agar, distributing the culture material throughout the agar by elevating the tube quickly from an inclined position, with a rotary motion.
 4. Flame the mouth of the second tube and pour the agar aseptically into a sterile Petri plate, raising the cover on one side only. Allow the agar to solidify before handling plate. When solid, inoculate the surface of the agar by drawing the loop filled with culture material gently over the surface in parallel streaks. Cover and invert plate.
 5. Store the gelatin tube at room temperature, in the desk locker. Place other cultures in tray in incubator room.
- B. Examine the cultures after incubation.
1. Make fresh preparation from bouillon tube, and stain film made from one of the cultures on solid media.
 2. Note character of growth on agar slant for (a) amount (b) consistency (c) pigmentation.
 3. Examine bouillon tube for (a) turbidity (b) pellicle (c) sediment.
 4. Note appearance of gelatin stab culture and form of liquified portion.
 5. Note size, appearance and distribution of colonies in agar tube culture.
 6. Study isolated colonies on agar plate with the unaided eye and with the low power of the microscope.
 7. Make drawings in note book to illustrate each culture.

EXERCISE 5

The Isolation of Bacteria: Aerobic Bacteria from the Air

Material Needed:

- A. 3 Deep agar tubes
3 Petri Plates
- B. 2 agar slants

One purpose of this exercise is to examine the possibilities of aerial contamination and the necessity for proper care in avoiding and detecting this source of error in bacteriological technic.

- A. 1. Pour three Petri plates of plain agar as in previous exercise. Allow the agar to solidify.
- 2. Remove the cover from one plate and expose the agar to the air for 5 minutes by the watch.
- 3. Expose the second plate to the air for 20 minutes.
- 4. Keep third plate unexposed as a control.
- 5. Students at odd desks keep plates in desk lockers, those at even desks place in tray in incubator room.
- B. 1. Study plates exposed to air and observe the different types of colony. It is one of the marks of the experienced bacteriologist to be able to judge the probable relationships of a bacterial culture by the microscopic appearance of well separated isolated colonies. In addition to the size of the isolate colonies, consistency, pigments, elevation, character of the edge, and other points may frequently be noted with value. See in appendix Chester's Terminology for description of Colonies p. 92.
- 2. With wax pencil make a circle on bottom of Petri plate about well isolated colonies, and place an identifying number beside each circle. Fish from these colonies to slides, make film and stain by Gram. The technic of fishing from colonies will be demonstrated by the instructor.
- 3. Inoculate agar slants from two colonies showing different types of organism, preferably one coccus and one bacillus.
- 4. Enumerate colonies on each of the plates. Tabulate observations.
- 5. Incubate subcultures at 37°C.
- G. 1. Study cultures fished from plates; make fresh and stained preparations to check the purity of culture.

EXERCISE 6

Isolation from Mixed Culture by Plating

Material Needed:

- A. Mixed culture in broth of *Micrococcus catarrhalis*
 Staphylococcus aureus
 Bacterium coli
- Five deep agar tubes
Five Petri plates
- B. Five plain agar slants
- A. 1. Pour plate method. Melt five tubes of agar in agate cup and cool to 45°C. Letter tubes a, b, c, d, and e. Inoculate (a) with one loop of mixed culture supplied. Mix thoroughly as in Ex. 4. Inoculate (b) from (a) with three loops. Mix. Inoculate (c) from (b) with five loops. Mix. The agar in all the tubes should be kept melted by standing between manipulations in water in the cup at 45°C. The contents of each tube, including the uninoculated control, (d) should be poured aseptically into a separate sterile Petri plate. Arrange the pour plates in a stack and place the agate cup on top of the stack. This will warm the cover of each plate and prevent condensation of water on it.
2. Streak plate method. Pour tube (e) of melted agar into a sterile Petri plate, allow the agar to harden. Take a loop of the mixed culture and make three closely parallel streaks along one side of the plate. Flame the loop and cover the remaining surface of the agar with parallel streaks one cm. apart at right angles to the previous ones, touching the inoculated portion and passing from it to the opposite side of the plate. Any similar method for spreading the mixture out in diminishing concentration will serve. The last portion streaked should show well separated colonies.
3. Invert all plates, label with wax pencil, and incubate at 37°C.
- B. 1. Study plates made at previous hour. The uninoculated control (d) should be sterile. If it is not, it indicates contamination due to faulty technic. One or more of the inoculated plates should show well separated colonies. On pour plates distinguish between deep and surface colonies. Sketch the different types of colony in each situation.
2. Mark with wax pencil on bottom of plate surface colonies sufficiently separated to permit of fishing. Make films and stain by Gram. Attempt to find each of the species present in the original mixed culture.
3. Fish under the low power of the microscope from one colony of each type to a plain agar slant. Incubate subcultures at 37° C.

4. Make blue-print of pour plate showing fewest colonies. See Broadhurst, Blue Printing Directly from Agar Plates. Jour. Bact. 3, 1918, p. 187.
- C. Examine cultures fished from plates. Test for purity of culture by fresh and stained preparations. Describe characteristics of each culture, and confirm previous findings as to staining reaction and morphology.

BIOCHEMICAL ACTIVITIES OF BACTERIA

EXERCISE 7

Oxygen—Carbon Dioxide Exchange (Respiration)

Material Needed:

24 Hr. culture of Bact. coli
Bacillus subtilis

3 bouillon tubes (5cc.)

Sterile vaseline

2 bouillon tubes with CO₂ trap

Phenol red indicator

M

NaOH — (carbonate free)
100

Paraffined corks

Oxygen indicator

(Indigo carmine 0.1%, K₂CO₃ 1%, Dextrose 1%. Allow to stand over night to permit reduction of the indigo.)

- A. 1. Inoculate tubes of bouillon with B. coli and B. subtilis. Cover surface of medium in these tubes and in uninoculated control tube with melted vaseline.

M

2. With capillary pipettes, add 5 drops to $\frac{M}{100}$ NaOH and 2

drops of phenol red indicator to the distilled water contained in the small CO₂ trap within the larger tube. Then inoculate bouillon in tube with B. subtilis. Plug the tube tightly with paraffined cork. Add NaOH and indicator in a similar way to the small tube of the uninoculated control, and plug tightly with cork. Do not flame mouths of these tubes or hold them near Bunsen flame at any time, as this would permit entrance of a large amount of CO₂.

- B. 1. Test for presence of oxygen in tubes treated according to directions in par. 1 of previous lesson. Take up a small amount of oxygen indicator (reduced indigo carmine) with a capillary pipette. Protect the indicator from air by drawing up first some vaseline, then the indicator, and again a small amount of vaseline to seal the tip of the capillary. The vaseline may be melted by placing the tube in a vessel of water at 40°C. Push the tip of the pipette through the vaseline covering the uninoculated control tube and inject half the indicator solution into the bouillon. With fresh pipettes inject indicator solution into the inoculated tubes. Avoid forcing even the smallest amount of air beneath the vaseline seal of the tubes to be tested.

In the presence of molecular oxygen the indicator solution immediately develops a bluish-green color.

2. Examine tubes containing CO₂ trap. CO₂ absorbed by the liquid in the small tube increases its acidity, as indicated

by change in color of the phenol red. What is the source of the volatile acid produced in the inoculated tubes?

REFERENCE

1. Efimov, V. V., Colorimetric Method for Oxygen. *Biochem. Z.*, 155, 1925, p. 371.
2. Novy, F. G., Roehm, H. R., Soule, M. H., Novy, F. G., Jr., Microbic Respiration. *J. Inf. Dis.*, 36, 1925, pp. 109, 168, 245, 343.
3. Coulter, C. B., and Isaacs, M. H. Reduction Potentials of the Typhoid Bacillus. *J. Exp. Med.* 49, 1929, p. 711.

EXERCISE 8.

Fermentation

A. *Material needed:*

- 24 hr. culture of *B. subtilis*
- B. lactis aerogenes*
- Yeast
- 2 dextrose agar slants
- 3 dextrose bouillon tubes (15cc.)
- 1 dextrose fermentation tube (Smith type)

1. Inoculate the dextrose agar slants with *B. subtilis*. Plug one tube tightly with parafined cork.

2. Inoculate two tubes of bouillon and the Smith fermentation tube with *B. lactis aerogenes* and one tube of bouillon with yeast. Incubate at 37°C.

B. *Material needed:*

- pH indicators Phenol Red
- Brom-thymol-blue
- Methyl Red

M/50 NaOH

Ether

Concentrated H_2SO_4

5% H_2SO_4

Saturated solution CuSO_4

Thiophene, 0.5% alcoholic solution

10% Na_2CO_3

Solution I_2KI

1% solution $\text{K}_2\text{Cr}_2\text{O}_7$

10% NaOH

1. Observe relation between oxygen supply and fermentation in agar cultures of *B. subtilis*. Make slide cover slip preparation from both tubes; allow a drop or two of Grams iodine to flow under cover slip. Examine with high-power dry lens and oil immersion for presence of glycogen granules in the bacilli. Are they present in preparations from both cultures?

2. Test cultures in bouillon for products of fermentation of dextrose.

a. *Organic Acids.*

Determine the pH of the culture, by adding 3 drops of each of the indicators to 3 cc. portions of culture and examining in the comparator block.

b. Titrate a 5cc. portion to pH 7.6 with phenol red and M/50 NaOH

Does the value found represent all the acid which has been produced in growth? See Exercise 7.

c. Test for lactic acid. The first part of this test should be done at one of the large tables, away from Bunsen flames. Shake out a 10cc. portion with 5 cc. ether. Place the tube in the refrigerator while the ether layer is separating. Pipette the ether layer into a dry test tube and evaporate on the water bath. Dissolve the residue in 2cc. of distilled water. Add 5 cc. conc. H_2SO_4 and 1 drop of saturated CuSO_4 and mix. Heat in boiling water 1 minute, cool under tap, add 2 drops of 0.5% alcoholic solution of Thiophene. Shake and replace tube in boiling water. A cherry-red color develops with lactic acid.

3. Test yeast culture for production of alcohol.

a. To $1\frac{1}{2}$ cc. portion of broth culture add a few drops of 10% Na_2CO_3 and 5 to 20 drops of I_2KI adding enough to render the mixture bright yellow for $\frac{1}{2}$ minute (Grams iodine solution may be used). Warm slightly, note odor of iodoform, and set aside. Examine the separated yellow iodoform crystals (rosettes) under the microscope. Acetone and other substances also give this reaction.

b. Distill off from a 10 cc. portion, using a bent glass tube and a rubber stopper, 3 cc. of distillate. Add 2-4 drops of Potassium dichromate, acidify with dilute H_2SO_4 and boil. Note odor of aldehyde, changing to that of acetic acid.

4. Test culture of *B. lactis aerogenes* for acetyl-methyl carbinol. To a 5 cc. portion add an equal amount of 10% NaOH and a few drops of H_2O_2 . Incubate for 24 to 72 hours. The development of a red color indicates the presence of acetyl methyl carbinol. The test is known as the Voges-Proskauer reaction. The significance is discussed by Levine, M., Jour. Bact., 1, 1916, p. 153.

5. Observe gas accumulation in closed arm of Smith fermentation tube. Mark the level of the liquid medium in this arm and with a scale measure the length of the column of gas. Add 10% NaOH to the open arm of the tube, filling it completely; place the thumb over the mouth of the tube and invert several times. Allow the remaining gas to collect in the closed arm and carefully remove thumb. Mark the level of the medium, measure the column of hydrogen and calculate the ratio between H_2 and CO_2 produced by the culture.

EXERCISE 9.

Protein Metabolism

The chemical changes considered in this exercise are not brought about by bacteria universally, and, on the other hand, represent only a few of the many chemical transformations which bacteria may produce in protein. The substances which are responsible for immune reactions, as well as the bacterial toxins, are products of protein or nitrogen metabolism but are too complex and of too uncertain nature to be studied here.

A. Material needed:

- 24. h. culture of *B. pyocyaneus*
B. sporogenes
B. coli

- 1 gelatin tube
- 1 Loeffler's serum slant
- 2 milk serum tubes (5 c. c.)
- 1 Dunham's peptone (10 c. c.)

1. Inoculate Loeffler's serum and gelatin with *B. pyocyaneus*. Make stab culture in gelatin and incubate at room temperature.

2. Heat milk serum tubes in boiling water for 15 minutes, cool rapidly and inoculate heavily with *B. sporogenes*.

3. Inoculate Dunham's peptone with *B. coli*.

B. Material needed:

- Ammonium sulfate (crystals)
- NaOH 10%
- CuSO₄ 0.1%
- Thymol blue indicator
- Lead acetate solution
- Ehrlich Indol reagents.

1. Examine cultures in gelatin and Loeffler's serum for liquefaction of the medium. Make observations over a period of 5 to 7 days. If the gelatin becomes liquified by melting, place in incubator for 24 to 48 hours, then test for ability of the medium to harden by placing in refrigerator.

2. Test culture in milk serum for proteolysis. Precipitate the proteins from a 5 c.c. portion by the addition of ammonium sulfate to saturation, and heat to 50° to 60°C. for thirty minutes. Filter through paper; to filtrate add strong NaOH and a few drops of dilute copper sulfate. A violet color indicates the presence of peptone, resulting from the proteolysis of the protein originally present.

3. Test culture in milk serum for ammonia liberated by deamination of amino acids. Moisten a plug of absorbent cotton with distilled water and 2 or 3 drops of thymol blue indicator, and push the plug into the culture tube to within 2 cm. of the surface of the medium. Place the tube in boiling water. Liberation of ammonia is indicated by development of the alkaline color in the indicator.

4. Add lead acetate to a culture in milk serum and test for liberation of S from sulphur-containing amino-acids.

5. Test the cultures in Dunham's peptone for the production of indol.

Moisten a small plug of absorbent cotton with the Ehrlich reagent and insert into tube containing 5 c.c. of culture; push the plug down to within 2 cm. of the surface of the liquid. Place tube in boiling water. Indol present is volatilized along with water and condenses in the cotton plug where it reacts with the reagent to give a reddish color.

EXERCISE 10

Oxidation-Reduction Activity

A. *Material needed:*

- 24 hr. cultures of *B. coli*.
 - B. phosphorescens*
 - Streptococcus viridans*
- 2 tubes of nitrate broth
- 1 tube of bouillon with indigo carmine
- 1 tube of infusion bouillon
- 1 tube of sea water agar.

1. Inoculate one tube of nitrate broth and the indigo carmine bouillon with *B. coli*.
2. Inoculate sea water agar with *B. phosphorescens*.
3. Inoculate infusion bouillon with *Strept. viridans*.

B. *Material needed:*

- Nitrate reagents (sulfanilic acid, naphthylamine)
- Dilute acetic acid
- Saturated ammonium sulfate solution
- 5% solution of sodium nitroprusside
- Concentrated ammonium hydroxide
- 1% solution α -naphthol in 95 % alcohol
- 1% solution paraphenylenediamine—HCl in water
- 1% sodium carbonate solution
- 3% suspension of washed sheep cells

1. Test the nitrate broth culture and the uninoculated control tube for the reduction of nitrate to nitrite as follows:

Transfer 4 c.c. of culture to a clean test tube. Add gradually 2 c.c. of freshly mixed nitrate reagent solutions. Place tube in warm water for 20 minutes. A pink color develops in the presence of nitrites. Observe promptly.

2. Observe reduction of indigo carmine in culture containing the dye. Agitate tube vigorously and note restoration of color on aeration.

3. Test for reduced glutathione in culture of *B. phosphorescens*. Transfer a quantity of culture to the concavity of a hollow-ground slide. Add dilute acetic acid and heat slide over flame until it begins to steam. Carefully decant acetic acid and add saturated ammonium sulfate. Change the latter solution twice. To the culture material in ammonium sulfate add 5 to 10 drops of sodium nitroprusside and allow to stand 3 to 5 minutes; then add concentrated ammonium hydroxide. A reddish color develops at once and fades in a few seconds, in the presence of sulfhydryl. Acetone as well as other substances also give the reaction.

4. Observe spectrum of reduced cytochrome in culture of *B. phosphorescens*. Transfer culture material from agar slant to a cell 3 mm. deep, illuminate with ribbon-filament bulb, using the substage condenser and examine through the microspectroscope.

5. Oxidation of Dextrose. Refer to Exercises 7 and 8.

6. Indophenol oxidase. Emulsify a quantity of *B. phosphorescens* culture in 5 c.c. bouillon in a test tube. Add 5 drops of 1% alpha naphthol, 5 drops of 1% paraphenylene diamine, and 5 drops of 10% sodium carbonate solution. Allow to stand 30 minutes. A reddish color, turning to purple develops on oxidation of the reagents by the oxidase.

7. Observe oxidation of oxyhemoglobin to methemoglobin by *Streptococcus viridans*. With capillary pipette place 1 c.c. of red cell suspension and 1 c.c. of bouillon culture of streptococcus into a small test tube. Allow tube to stand in water bath at 37° C. for one half to one hour. A greenish brown color develops in the erythrocytes as the oxyhemoglobin becomes oxidized to methemoglobin.

EXERCISE 11

Differential Hydrolysis of Carbohydrates

Material needed:

- 24 hr. cultures of *B. coli*
 - B. paratyphosus* A.
 - B. alkaligenes*
 - B. subtilis*
- 1 tube of dextrose agar
- 1 " " lactose "
- 1 " " saccharose "
- 1 " " mannit "
 - 15 c.c. per tube, containing andrade indicator
- 1 tube of starch agar.
- Solution of iodine in 50% ethyl alcohol

- A. Melt the agar containing the different carbohydrates and pour Petri plates. When cool, inoculate each plate with each of the organisms supplied, making a single streak across the plate. Number or otherwise identify each streak on the bottom of the plate. Incubate at 37°.
- B. 1. Examine cultures for acid produced by the fermentation of the dextrose resulting from hydrolysis of the sugars. Record the results in a table.
2. With a pipette, place 4-5 drops of solution of iodine upon the starch-agar plate, and allow the iodine to flow at right angles across the culture streaks. Observe reaction, and note clearing of medium about portions of growth not treated with iodine.

EXERCISE 12

Production of Pigment and Light

Material needed:

24 hr. cultures of *Staphylococcus aureus*
 B. prodigiosus
 B. pyocyaneus
 B. phosphorescens

3 agar slants

1 tube of broth

1 tube of sea-water broth

- A. 1. Inoculate *Staphylococcus aureus*, *B. prodigiosus* and *B. pyocyaneus* on dextrose agar slants.
2. Inoculate *B. pyocyaneus* into broth.
3. Inoculate the sea-water broth with *B. phosphorescens*.
4. Incubate all cultures at room temperature in the dark.
- B. 1. Observe presence, color and distribution of pigment in cultures.
2. Examine broth cultures of *B. pyocyaneus*. Agitate tube vigorously and observe appearance or deepening of color. Allow the tube to stand, and note any change in appearance of pigment. Offer an explanation for the change.
3. Shake out culture of *B. pyocyaneus* with 3 c.c. of chloroform. Note solubility of pigment in this solvent. Pipette off the aqueous layer and evaporate the chloroform solution to dryness on the water bath.
4. Test solubility of the dry pigment in water, alcohol and ether.
5. Make similar preparations of pigment from cultures of other bacteria, and apply the lipocyan test to the dry pigment, as follows: Add 1 drop of concentrated H_2SO_4 and observe for production of intense blue color. This reaction is given only by the pigments known as carotinoids from their resemblance to plant carotin.
6. Carry the culture of *B. phosphorescens* into a dark room. Allow the eyes to become accustomed to the darkness and examine the culture for luminescence. Agitate the tube and note any change in the production of light.
7. Add a few drops of $\text{K}_3\text{Fe}(\text{CN})_6$ solution to the luminescent culture. Observe the tube in the dark. Does it luminesce when agitated?

REFERENCES:

- Buchanan and Fulmer, *Physiology & Biochemistry of Bacteria*. Vol. 1, Chapter III.
Harvey. *The Nature of Animal Light*.
Lippincott, Philadelphia, 1920.
Morrison, L. F., *Studies in Luminous Bacteria*. *Jour. Gen. Physiol.*, 7, 1925, p. 741.

PATHOGENIC MICRO-ORGANISMS

Throughout the rest of the course we concern ourselves largely with microorganisms in their relation to disease.

No attempt is made to study all pathogenic microorganisms in this course. Only the most representative and important species are considered as illustrative of general principles or of particularly important details. Some of the most important diseases of known animate causation cannot readily be studied owing to technical difficulties that would require too much time or skill for the student to master. The ingenuity of each individual may be indulged in connection with the assigned problems which now assume significance.

It is desirable that the student should learn to think of the pathogenic bacteria in their position in natural taxonomic groups as well as in their relation to specific diseases. Practical utility in bacteriology has out-stripped strictly scientific classification; interest has centered in the pathogenic members of a given genus rather than in an attempt to collect all possible related members of it. Efforts are now under way, however, to classify bacteria in a more exact fashion. On page 190 will be found a summary of Bergey's Manual of Determinative Bacteriology, simplified to cover the medical aspects of bacteriology.

The ideal source of material for the study of pathogenic bacteria is diseased tissue from which they are originally isolated. Whenever possible such material in the form of pus, secretions, blood, urine, feces, or tissues from human beings or animals will be furnished. Stock cultures are used in other instances.

Exercises in infection and immunity are presented in connection with the exercises on pathogenic bacteria. These exercises will be assigned to small groups of students, and will be undertaken as original problems and reported to the class at large. Knowledge of the scientific background of the subject illustrated by the experiment is expected, but only the student's experimental observations should be reported, leaving to general discussion the formulation of appropriate theory and the development of ideas regarding the significance of the experiment.

Brief statement only of the object and method of each exercise is given. The student should seek more complete information in the references to the literature of the subject which are given. In every instance a plan of experimentation should be submitted to the instructor for approval after references have been read and before the experimental work is started.

EXERCISE 13

Genus Staphylococcus

Usually parasitic. Cells in irregular groups, rarely in packets. Usually Gram-positive. Growth good as a rule on artificial media. Gelatin may be liquified, nitrates may be reduced. Pigment produced: white, lemon yellow or orange.

S. aureus is the principal pathogenic member. It is the most common organism causing suppuration.

A. *Material needed:*

- 24 hr. culture of *S. aureus*
- Material from infected animal
- 2 agar plates
- 1 tube gelatin
- 1 tube bouillon

1. Make fresh and stained preparations of culture and of material from infected animal.

2. With stock culture of *S. aureus* streak one agar plate and inoculate gelatin and bouillon tubes. Incubate gelatin tube in desk locker.

3. Streak out material from infected animal on the other agar plate.

B. *Material needed:*

- 2 agar slants
- 3 bouillon tubes
- 1 blood agar plate

1. Examine plate made from stock culture of *S. aureus*; mark isolated colonies. Fish from these and examine stained films using methylene blue and Gram stains, and make subcultures to agar slant and bouillon tube.

2. Carry 2 loops of bouillon culture of *S. aureus* over to sterile tube of bouillon, and carry over 3 loops from this to tube of melted agar at 45° C. Add 1 c.c. of sterile blood, mix and pour plate.

3. Examine agar plate inoculated with material from infected animal for typical colonies. Fish from isolated colonies to agar slant and bouillon tube.

1. Compare cultures made from stock strain of *S. aureus* and from animal material. Make fresh and stained preparations. Note character of growth and grouping of organisms in bouillon culture and examine agar slants for pigment formation.

2. Examine gelatin tube from A-2 for liquefaction.

3. Examine blood agar pour plate for hemolysis.

REFERENCE:

- 1. Neisser Die Staphylokokken. Kollé, Kraus and Uhlenhuth.
- 2. Buchanan Studies in the Nomenclature and Classification of the Bacteria. Jour. Bact., 2, 1917, 603.
- 3. Julianelle Studies of Hemolytic Staphylococci. Jour. Inf. Dis., 31, 1922, 256.

INFECTION AND IMMUNITY

1. Methods of obtaining whole blood, red corpuscles, leucocytes, and blood serum. A variety of methods are in use. Students will learn under the guidance of the instructor and will demonstrate to the class the methods of obtaining blood from laboratory animals and separating cells and serum.

As general references consult

Kolmer

Zinsser, Hopkins and Ottenberg

2. Formation of sterile exudates.

a. Serous exudate. Place ear of rabbit in water at 56° C. for 3 minutes. Follow course of reaction for 48 hours.

b. Purulent exudate. Rub drop of croton oil into ear of rabbit. Follow course of reaction for 48 hours.

REFERENCE :

Maccallum.

3. Local abscess.

Inoculate a rabbit intradermally with culture of *S. aureus*. Examine inoculated area daily for production of lesions and for presence of staphylococcus.

REFERENCE :

Maccallum.

4. Pyemia.

Inoculate a rabbit intravenously with a culture of *S. aureus*. Make daily observations on temperature and weight of the animal and make cultures of blood. Autopsy. Examine internal organs in the gross and in microscopic section for lesions and make cultures to determine presence of *S. aureus*.

REFERENCE :

Kolmer.

5. Preparation of a Bacterial Vaccine.

Secure a pure culture of *S. aureus* by isolation. Prepare vaccine by the method given in Zinsser. Kill by minimum exposure to heat, with consideration of the thermal death point of the microorganism. Test for sterility of the preparation. Standardize in two different ways.

Zinsser, Hopkins and Ottenberg, p. 176.

Wadsworth, p. 457

Jordan and Falk, p. 285.

6. Leucocytosis.

Inject intravenously into rabbits sterile milk and bacterial vaccine. Make leucocyte counts hourly and observe the stages of leucopenia and hyperleucocytosis.

REFERENCE :

Stitt

Zinsser, Infection and Resistance.

TRIBE STREPTOCOCCUS

Cells spherical or hemispherical. Planes of fission parallel, resulting in pairs or chains of cells. Generally Gram positive. Essentially parasitic, except *Leuconostoc*, and growth not abundant as a rule on artificial media.

EXERCISE 14

GENUS STREPTOCOCCUS

Chiefly parasites. Fresh cultures not dissolved by bile. Characteristic reactions on red blood cells.

The streptococci produce a wide variety of infections. The most common locations in which the streptococcus is found are external wound infections, erysipelas, severe acute infections of the nasopharynx and neighboring structures, scarlet fever, the lung and pleura in certain forms of pneumonia and the blood in streptococcus bacteriemia.

A. *Material needed:*

- 24 hr. culture of *Strept. hemolyticus*
Strept. viridans

Material from experimental animal infected with *Strept. hemolyticus*
2 blood agar plates

1. Make fresh and stained preparations of stock cultures and of material from infected animal.

2. Divide one blood agar plate into two portions by marking on the bottom with a wax pencil. On one portion of the plate streak out the culture of streptococcus hemolyticus, on the other streak out streptococcus viridans.

3. Streak out material from infected animal on the second blood agar plate. Incubate cultures at 37°C.

B. *Material needed:*

- 3 bouillon tubes
- 1 dextrose agar plate

1. Study plate culture of streptococcus hemolyticus and streptococcus viridans. Observe characteristics of isolated colony, and examine for hemolysis or methemoglobin formation about colonies.

2. Fish from isolated colonies of each form of streptococcus; inoculate plain and dextrose bouillon tubes and streak out on a portion of dextrose agar plate.

3. Examine plate culture of material from infected animal for colonies typical of streptococcus hemolyticus. Fish from isolated colonies to bouillon tube and to a portion of dextrose agar plate.

C. *Material needed:*

- pH indicators
- Wasserman tubes

- 3% washed red blood cell suspension
- Sterile bile or bile salt solution
- Water bath at 37° C.

1. Observe character of growth in bouillon cultures of streptococci. Examine by fresh and stained preparations, making preparations from sediment in tubes and from supernatant fluid.

2. Study agar plate cultures with hand lens and low power microscope and observe colony characteristics.

3. Test for fermentation of dextrose by pH change in dextrose bouillon cultures.

4. From plain bouillon cultures of each type of streptococcus, pipette 15 drops into each of two small test tubes. To the first add 10 drops of 3% red blood cell suspension, to the second tube 5 drops of sterile bile or bile salt solution. Prepare control tubes of blood plus sterile bouillon, and culture plus sterile bouillon. Place in water bath one hour. Examine for hemolysis, methemoglobin formation and solution by bile.

Carry out appropriate tests to identify organism isolated from infected animal as streptococcus hemolyticus.

REFERENCES :

Gay—Recent Aspects of Streptococcus Infections. Jour. Lab. and Clin. Med., vol. 3 (1918), p. 3. Methods for the Isolation and Identification of S. hemolyticus. Adopted by the Medical Department, U. S. Army, 1918.

Holman—The Classification of Streptococci. Jour. Med. Res., vol 34 (1916), p. 377.

Foster—The Biochemistry of Streptococcus hemolyticus. Jour. Bact., vol. 16 (1921), p. 211.

Dochez, Avery and Lancefield—Studies on the Biology of Streptococcus. I. Antigenic Relationships between Strains of Streptococcus hemolyticus. Jour. Exp. Med., vol. 30 (1919), p. 179.

Lancefield, R.—Antigenic Complex of Streptococcus Hemolyticus. J. Exp. Med., 48, 1928, 91, 469, 481.

Dochez, A. R.—Etiology of Scarlet Fever. Medicine, 4, 1925, 251.

Dick & Dick—Results with Skin Test for Susceptibility to Scarlet Fever. J. A. M. A., 84, 1925, 1477.

7. Septicaemia.

Inoculate rabbits intravenously with pathogenic strain of streptococcus hemolyticus. Follow course of infection by observations of temperature, weight, and blood cultures. Autopsy. Make culture from hearts blood and from organs.

Hopkins and Parker, Jour. Exp. Med., 27, 1918, p. 1.

Zinsser, Infection and Resistance.

8. Sero-Fibrino-Purulent Exudate.

Produce streptococcus empyema in a rabbit by intrapleural inoculation. Examine exudate daily by thoracentesis. Follow course of temperature and weight, and take blood cultures. Autopsy. Examine pleurae in the gross and in microscopic section, and make cultures.

Gay and Stone, J. Inf. Dis., 26, 1920, p. 268.

Gay and Morrison, J. Inf. Dis., 33, 1923, P. 337.

EXERCISE 15

Genus Neisseria (Gram Negative Diplococcus Group)

Strict parasites, failing to grow or growing very poorly on usual artificial media. Cells normally in pairs, occasionally in tetrads. Gram negative.

The pathogenic members of this group are: (1) *Neisseria intracellularis* or meningococcus, the organism causing epidemic meningitis and found in the inflamed meninges and blood stream of patients, and in the naso-pharynx of patients and healthy carriers; (2) the *Neisseria gonorrhoeae*, or gonococcus, causing urethritis and less commonly conjunctivitis and arthritis. True infection in the ordinary laboratory animals is not possible and stock cultures are the source of class material. It should be borne in mind in connection especially with this and the following group of bacteria, that in becoming adapted to culture media the bacteria have lost many of the characteristics of freshly isolated strains.

A. Material needed:

- 24 hr. cultures of meningococcus
- gonococcus
- micrococcus catarrhalis
- Films of pus from urethritis
- 3 deep dextrose agar tubes
- 2 c.c. sterile ascitic fluid
- 2 c.c. sterile laked blood
- 3 Petri plates

1. Melt agar and cool to 45°. Pour plates of simple dextrose agar, ascitic fluid agar and laked blood agar, mixing the ascitic fluid and the blood thoroughly with the agar as directed in a previous exercise.

2. Divide each plate into three compartments with the wax pencil. Make streak inoculations in one compartment of each plate with each of the cultures supplied. Incubate at 37°.

3. Make fresh and stained preparations of the stock cultures, using both methylene blue and Gram stains, and stain the films of urethritis pus supplied. Note characteristics of morphology and staining reaction, and grouping of cocci in pus films.

B. Material needed:

- Saline suspension of meningococcus (killed)
- Anti-meningococcus serum 1/20
- Normal horse serum 1/20
- 3 Wassermann tubes
- Water bath at 55°
- 2 dextrose ascitic agar slants, with Andrade or litmus indicator
- 2 maltose ascitic agar slants, with Andrade or litmus indicator

1. Study plate cultures. Note characteristic differences in growth of the different organisms. Examine isolated colonies with the hand lens, as well as with the low power microscope. Attempt to identify the meningococcus by its colony characteristics, especially on the laked blood medium.

2. Inoculate the meningococcus and gonococcus upon dextrose and maltose fermentation agar slants. Incubate at 37°.

3. Examine stained films of growth on plates. Repeat Gram stain if necessary to show characteristic staining reaction.

4. With capillary pipette place 15 drops of saline emulsion of meningococcus into each of three small tubes. To the first tube add 15 drops of saline, to the second tube 15 drops of normal horse serum diluted 1/20 and to the third 15 drops of antimeningococcus serum diluted 1/20. Place tubes in waterbath at 55° for thirty minutes. Agitate tubes by flicking with the finger after the first five minutes incubation. Examine tubes for agglutination, using the hand lens if gross agglutination is not apparent.

REFERENCES :

Meningococcus

Elser and Huntoon—"Studies on Meningitis." Jour. Med. Res. (1909), vol. XX, p. 371.

Flexner—Mode of Infection, Means of Prevention and Specific Treatment of Epidemic Meningitis. Jour. A. M. A., vol. 69 (1917).

Foster and Gaskell—Cerebro-Spinal Fever. Cambridge University Press, England. 1916.

Gonococcus:

Cole and Lloyd—Preparation of Solid and Liquid Media for the Cultivation of the Gonococcus. Jour. Path. and Bact., vol. 21 (1917), p. 267.

Cooke and Stafford—A study of the Gonococcus and Gonococcal Infection. Jour. Infect. Dis., vol. 29 (1921), p. 561.

Torrey and Buckell—A Serological Study of the Gonococcus Group. Jour. Immunology, vol. 7 (1922), p. 305.

INFECTION AND IMMUNITY

9. *Phagocytosis*. The action of leucocytes on bacteria. Prepare the peritoneal cavity of a guinea pig by injecting sterile broth or aleuronat-starch mixture into the cavity. On the following day inject intraperitoneally a small quantity of a broth culture of a coccus. Withdraw small amounts of fluid from the peritoneum at 15 minute intervals. Prepare films, fix with methyl alcohol and stain by Wright stain.

10. Action of macrophages on nucleated red blood cells. Inject suspension of washed pigeon or hen erythrocytes into the peritoneal cavity of a guinea pig which has been prepared by the injection of gum-arabic beef extract broth 48 hours previously. Withdraw fluid

at 15 minute intervals. Stain fresh preparations with neutral red; Prepare films, fix with methyl alcohol and stain with Wright stain.

REFERENCES:

Kolmer, p. 1121

Gay and Morrison—J. Inf. Dis., 33, 1923, 338

Gay and Clark—Arch. Path. & Lab. Med., 1, 1928, 847

Zinsser—Infection and Resistance

11. Mechanism of Action of Opsonin. Obtain pleural exudate from a rabbit, suspension of cocci (*Meningococcus* or *Pneumococcus*), and normal and immune horse or rabbit sera.

(a) Demonstrate difference in phagocytosis due to presence of normal and immune opsonin.

(b) Determine whether the action of opsonin is on the bacteria or on the leucocytes.

REFERENCES:

Zinsser et al, p. 168

Kolmer, p. 1171

EXERCISE 16

Genus Vibrio

Short slightly curved rods, single or united into spirals. Motile, 1 to 3 polar flagella. Gram negative.

Vibrio cholerae is the principal member and probably the only one of the group that is pathogenic for man. The organism is found in the intestinal canal of patients and carriers.

A. *Material needed:*

- 24 hr. cultures of *V. cholerae*
V. metschnikovi
- 2 deep agar tubes
- 2 Petri plates

1. Pour agar plates. Make streak inoculations on the surface of the medium from the cultures of two types of *Vibrio*. Care should be taken to make the inoculation sufficiently light.

2. Make stained and fresh preparations from stock cultures. Examine for motility.

B. *Material needed:*

- 2 gelatin tubes
- 2 alkaline agar slants
- 2 Dunham's peptone solution

Study agar plates. From isolated colonies of each organism make inoculation on the test media.

1. Examine gelatin tube for liquefaction.
2. Test Dunham's peptone solution for indol production.
3. Examine growth on alkaline agar medium. Compare with growth of *B. coli* on the same medium.

In practical work the two sources from which the isolation of the cholera vibrio is usually attempted are water, and the stools of patients and convalescents. With either of these materials preliminary enrichment by growth in peptone water is of advantage as, during the first five to eight hours of incubation, the cholera vibrio outgrows other forms in this medium and accumulates in a pellicle on the surface. This pellicle is then plated out on gelatin or Dieu-donne's alkaline agar.

REFERENCE:

Teague and Travis—*Jour. Inf. Dis.* 18, 1916, p. 601.

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12. Bactericidal Action of Normal Serum.

Demonstrate the bactericidal action of normal serum. Follow the method described in Zinsser, Hopkins and Ottenberg, p. 20, but use *V. metschnikovi* instead of *B. typhosus*.

REFERENCE:

Zinsser et al., p. 20.

Kolmer, p. 357.

13. Bacteriolysis. The Pfeiffer phenomenon in vitro.

Immunize a rabbit against *V. cholerae*. Assist instructor in trial bleeding and titration. Read Bordet and Gay, p. 56, and Kolmer, pp. 385 and 1151, on the Pfeiffer phenomenon. Outline a method for demonstrating this phenomenon in vitro, with rabbit anticholera serum and guinea pig alexin. Demonstrate bacteriolysis by the use of dilution plates and by films stained with dilute carbol fuchsin.

14. Normal Hemolysin. Demonstrate the hemolytic power of normal blood serum for foreign cells, using serum and cells of laboratory animals.

REFERENCE:

Zinsser et al., p. 22.

Kolmer, p. 394.

Kolmer & Casselman, J. Inf. Dis., 16, 1915, 441.

15. Experimental Hemolysin. Immunize a rabbit against sheep erythrocytes. Make a trial bleeding and test the hemolytic power of the serum. Make final bleeding when rabbit is sufficiently immunized.

Demonstrate

- (a) Dual nature of hemolytic action.
- (b) Specificity of hemolytic serum.
- (c) Union of sensitizer with cells.

REFERENCES:

Bordet & Gay, p. 134.

Zinsser et al., pp. 26, 34, 46.

Zinsser, Infection and Resistance.

EXERCISE 17

Genus Proteus

Pleomorphic rods. Motile, flagella peritrichous. Gram negative. Dextrose and saccharose fermented, not lactose. Actively proteolytic and occasionally hemolytic.

The proteus group is widely distributed in nature, and represents the common organisms of aerobic putrefaction. Its chief importance in medical bacteriology is its frequent occurrence as a contaminant in cultures, especially from organs at autopsy. It appears infrequently as a pathogen of considerable virulence in deep-seated abscesses. It is as a rule easily identified by the amoeba-like rapidity spreading colonies which it develops on moist solid media.

Genus Pseudomonas (Fluorescent Group)

Slender rods, actively motile. Gram negative. Carbohydrates utilized. Proteolytic. Characteristic pigment produced.

B. pyocyaneus is the only member of the chromobacteriae of pathogenic significance. It is encountered essentially as a saprophyte in suppurative processes due to other organisms. Its independent pathogenicity is apparently very low. Its persistence in wounds and hospital wards is remarkable.

A. *Material needed:*

24 hr. cultures of *B. proteus*

B. pyocyaneus

3 gelatin tubes

3 Petri plates

1 bouillon tube

1. Make dilution pour plates in gelatin. Alternate students may cultivate *B. proteus* and *B. pyocyaneus* and study their neighbor's cultures. Incubate at room temperature.

2. Inoculate bouillon tube.

3. Make fresh and stained preparations from the stock culture.

B. *Material needed:*

Wasserman tubes

5% suspension of washed sheep cells

Water bath at 37°

1. Study cultures in gelatin plates. Note characteristics of colonies, pigment production and action on gelatin.

2. Examine fresh and stained preparations.

3. Set up hemolytic test in tubes, as in previous exercises, using bouillon culture. Read after 30 minutes at 37°

REFERENCES:

- Bengston—The *Proteus* Group of Organisms. Jour. Inf. Dis., 24, 1919, p. 428.
Gordon—*Bacillus Pyocyaneus* and Its Pigments. Jour. Exp. Med., 4, 1899, p. 627.
Tanner—A Study of Green Fluorescent Bacteria from Water. Jour. Bact., 3, 1918, p. 63.

EXERCISE 18

Bacteriology of Water

Specimens of water for analysis should be collected in clean bottles sterilized by dry heat or in the autoclave. Care should be taken that the sample is representative of the water in question. It should be examined within 5 to 6 hours after collection. Samples are sometimes preserved for longer periods on ice but the results from their examination are unreliable.

The facts usually ascertained are:

(a) The number of bacteria present viable at 37°C (agar plating) and 20°C (gelatin plating.)

(b) The presence or absence of colon bacilli (presumptive lactose broth test.)

In addition the Standard Methods of Water Analysis requires:

(c) The isolation and identification of the lactose fermenters where (b) is positive. (Endo or eosin methylene blue plates.)

The amount used for examination varies from 10 c.c. for potable water to .000001 c. c. for fresh sewage.

A. Material needed.

Specimen of water (for class purposes inoculate 2 loops of a 24 hour broth culture of *B. coli* into 300 c.c. of tap water.)

2 sterile test tubes

Flask of sterile water

3 agar tubes for plating

2 Lactose fermentation tubes 5 c.c.

1 Lactose fermentation tube 30 c.c.

Pipettes: one 10 c.c.

two 1 c.c.

1. Test for bacteria viable at 37°C.

Into two sterile test tubes measure 9 c.c. of sterile water with a sterile pipette.

Label three Petri dishes 1 c.c., 0.1 c.c. and .01 c.c.

Shake specimen of water to be examined thoroughly.

With the sterile pipette measure 1 c.c. into first Petri dish and 1 c.c. into first test tube. Mix the dilution in the first test tube thoroughly by drawing in and blowing out of pipette and measure 1 c.c. from first test tube into second Petri dish and 1 c.c. into second test-tube. Repeat mixing procedure as for first tube and measure 1 c.c. from second test tube into third Petri dish. Into each Petri dish pour tube of agar melted and cooled to 40-50 degrees. Rotate carefully to secure an even mixture of the agar and specimen. Incubate inverted.

2. Presumptive Test for *B. coli*.

Shake specimen bottle. With sterile pipette inoculate three lactose broth fermentation tubes with 10 c.c., 1 c.c. and 0.1 c.c. of specimen respectively. There must always be at least three times as much medium in the tubes as the quantity of sample added. When 10 c.c. or more sample is used large tubes of lactose broth must be provided.

B. 1. Select plate containing from 30 to 300 colonies from set made at previous lesson. Count number of colonies and estimate number of bacteria in water examined.

2. Examine lactose broth tubes for presence of gas and estimate number of colon bacilli in specimen examined.

Verification of this test may be made by plating out the gas-containing cultures on Endo or eosin methylene blue medium, isolating the lactose fermenting organisms in pure culture and then carrying out cultural tests for its identification as a colon bacillus. Further tests which are necessary to interpret the sanitary significance of the lactose-fermenting organism in water are:

1. Methyl red test.
2. Voges-Proskauer test for acetyl-methyl carbinol.
3. Liquefaction of gelatin.
4. Fermentation of adonite.

These tests are applied to aid in determining whether or not the microorganism is of fecal origin.

REFERENCE:

Standard Methods for the Examination of Water and Sewage. Fifth Ed. 1927. Am. Public Health Assn., 370 Seventh Avenue, New York.

EXERCISE 19

Genera Escherichia and Eberthella (Colon-Typhoid-Dysentery Group)

Motile or non-motile rods occurring in the intestinal canal. When motile the flagella are peritrichous. Attack numerous carbohydrates forming in some cases abundant gas. Do not form acetyl-methyl-carbinol. Non-proteolytic.

This group includes the common *Bacterium coli*, which occurs normally in the human intestine, and may appear as a pathogen of slight or considerable virulence. Characteristic differences in fermentation reaction are observed between this form and the more highly pathogenic members which are found in patients and carriers. The correspondence of specific pathogenicity and immune reactions with carbohydrate fermentation is close, although not rigid, and fermentation reactions are relied upon for primary isolation and provisional diagnosis. Each species probably comprises a group of varieties or types separable only by immune reactions.

A. *Material needed:*

- 24 hr. cultures of *B. coli*
 - B. typhosus*
 - B. paratyphosus* A.
 - B. paratyphosus* B.

- 1 dextrose agar plate
- 1 lactose agar plate
- 1 saccharose agar plate
- 1 endo plate
- 1 eosin-methylene blue plate

1. Make fresh and stained preparations from the stock cultures.
2. Make parallel streaks of the stock cultures on each of the sugar plates, and on the endo and eosin-methylene blue plates.

B. *Material needed:*

- 1 tube of bouillon 10 c.c.
- 3 endo or eosin methylene blue plates

1. Observe characteristics of growth and fermentation reactions of cultures on the sugar plates and on the endo and eosin methylene blue plates. Attempt to distinguish the typhoid group of bacilli from *B. coli*.

2. Make Gram stain of each type of organism.

3. Emulsify a small portion of growth, taken up with the straight needle, from *B. coli*, *B. typhosus* and *B. para* A in the same tube of bouillon. Mix well and place two loopfuls of the mixture on an endo plate. Distribute the inoculum evenly over the surface of the plate with a glass streaking rod, and inoculate the second and third

plates in succession with the material adhering to the rod.

To prepare the glass spreading rod, soften a piece of glass rod at a point 3 cm. from the end, and allow the terminal portion to fall under its own weight until it makes an angle of 30° with the main portion. Sterilize each time before use by dipping in alcohol and allowing the alcohol to burn off. Sterilize after use by allowing to stand in lysol or other disinfectant solution.

C. *Material needed:*

4 Russell double sugar tubes

1. Study endo plates inoculated with the colon-typhoid-paratyphoid mixture. Distinguish lactose-fermenting from non-lactose-fermenting colonies.

2. Fish from one isolated colony of *B. coli* type and from three non-fermenting colonies to Russell double sugar tubes. Inoculate the surface of the slant by streak and the butt by stab.

D. *Material needed:*

1 Dextrose fermentation tube (Durham)

1 Lactose fermentation tube (Durham)

1 extract broth tube

1 Dunham's peptone solution.

1 Lead acetate agar

1. Observe Russell double sugar tubes for characteristic fermentation reaction.

2. From a slant showing typical typhoid or paratyphoid fermentation and giving Gram negative staining reaction, inoculate the test media.

E. *Material needed:*

Anti-typhoid and anti-paratyphoid agglutinating sera

Agglutination tubes and racks

Water bath at 37° C.

1. Observe fermentation tubes for formation of acid or gas.

2. Examine lead acetate agar culture for production of hydrogen sulfid.

3. Make fresh preparations from broth culture and examine for motility.

4. Test the Dunham's peptone solution for indol.

5. Heat the extract broth culture for 30 minutes at 60° . Then prepare two series of 4 tubes each, and pipette 10 drops of the broth culture into each of the tubes. In the first series, to the first tube add 10 drops of anti-typhoid serum diluted $1/25$, to the second tube 5 drops, and to the third, 1 drop. The final dilutions of serum are, therefore, $1/50$, $1/100$ and $1/500$. Make up the volume of all tubes, including the fourth tube, to 20 drops, with saline solution. Treat

the second series of tubes in the same way, using anti-paratyphoid A serum instead of anti-typhoid serum. Incubate the tubes at 37° for 30 minutes. Examine for agglutination.

6. Identify the organism isolated from endo plates on the basis of its fermentative and agglutinative characteristics.

REFERENCE:

Gay—Typhoid Fever. MacMillan, New York, 1918.

INFECTION AND IMMUNITY

16. *Agglutination and the Production of Agglutinin.*

Immunize rabbits against

- B. typhosus
- B. paratyphosus A.
- B. paratyphosus B.
- B. dysenteriae Shiga
- B. dysenteriae Flexner

When serum has been found after trial bleeding to be of sufficiently high titer, draw blood aseptically. With the serum obtained set up tests for agglutination of the homologous organisms and for cross agglutination of biologically related strains.

REFERENCES:

- Gay & Fitzgerald, Univ. Calif. Pub. Path., 2, 1912, 77.
Locke, *ibid.*, 2, 1912, 91.
Kolmer, p. 1129.

17. *Absorption of Agglutinin.*

(a) Absorb agglutin with each antigen separately from serum of rabbit immunized simultaneously against B. typhosus and B. paratyphosus. Titrate serum after absorption for removal of agglutinin.

(b) Absorb antityphoid serum with typhoid and with paratyphoid antigen. Titrate serum before and after absorption with each antigen.

REFERENCES:

- Castellani.
Zinsser et al., p. 74.
Krumwiede, Cooper & Provost. J. Immun., 10, 1925, 55.

18. *Experimental Typhoid Carrier.*

Prepare several experimental rabbit typhoid carriers, following directions given by Beckwith, J. Inf. Dis., 31, 1922, 468. Observe these rabbits for several weeks; autopsy at varying intervals and culture the bile for typhoid bacilli.

REFERENCES :

Gay & Claypole, Arch. Int. Med., XII, 1913, 613.
Gay—Typhoid Fever.

19. *The Typhoidin Test.*

Typhoidin will be supplied, and tests will be carried out on students who volunteer. They will be divided into groups as follows:

- (1) Those who have not had typhoid, or been vaccinated against typhoid.
- (2) Those who have been vaccinated against typhoid.
- (3) Those who have had typhoid.

The reactions of the members of these three groups will be compared.

Gay—Typhoid Fever.

EXERCISE 20

Dysentery Bacillus Group. (Eberthella Dysenteriae and Paradyserteriae)

This group comprises a number of closely related organisms which are differentiated both by fermentation and agglutination tests. The organisms are found in the stools of patients and carriers.

A. *Material needed:*

Suspension of moist feces seeded with:

B	dysenteriae	Shiga
"	"	Flexner
"	"	Y

2 Endo tubes

1 deep tube of dextrose agar

3 Petri plates

Pour plates. Inoculate the media with the fecal suspension using the spreading rod which is dipped once only into the mixed culture. Inoculate first one Endo plate then the agar plate, and then the second Endo plate. Incubate at 37°. If possible examine the plates at the end of the day, mark all colonies which have appeared and return to incubator.

B. *Material needed:*

4 Russell double sugar tubes

1. Study agar and Endo plates: distinguish dysentery colonies. Those colonies which have appeared after the colonies marked at the end of the previous day are more likely to be those of the dysentery

bacillus. From non-lactose fermenting colonies, fish to Russell double sugar tubes.

C. *Material needed:*

- 1 dextrose fermentation tube
- 1 mannit " "
- 1 maltose " "
- 24 hr. cultures of *B. dysenteriae* Shiga
 " " Flexner
 " " Y
- 1 bouillon tube
- 1 peptone water tube

1. Stain films and make fresh preparations. Examine for motility. Are the bacilli motile?

2. From Russell double sugar tube giving characteristic dysentery fermentation, inoculate the test media. If isolation was not successful, inoculate with one of the stock cultures.

D. *Material needed:*

- Anti dysentery agglutinating sera, diluted 1/100, corresponding to three types of bacilli
- Wassermann tubes

1. Note results of fermentation reactions in test sugars.

2. Test peptone water culture for indol production.

3. Set up agglutination test. Each group of four students will set up one series of tubes and will test their own culture and that of each of the neighboring group of students against one of the type sera supplied. Take up materials with capillary pipettes. Use the bouillon culture for the bacterial suspensions.

- Tube 1—15 drops diluted serum and 15 drops own culture
- 2— " " " " " 15 drops neighbor's culture
- 3— " " " " " " " "
- 4—15 drops saline solution and 15 drops own culture

Read agglutination after 30 minutes at room temperature.

4. The instructor will make chart of fermentation and serum reactions of the members of this group from data supplied by the students.

REFERENCES:

- Kliger and Olitsky—Method for the Isolation and Rapid Identification of Dysentery Bacilli. Jour. A.M.A. 1918, p. 2126.
- Kolle & Hetsch—Die Experimentelle Bakteriologie. Ed. 7, 1929, vol. 1.
- Davison—Divisions of the So-called Flexner Group of Dysentery Bacilli.

Jour. Exp. Med. XXXII, 1920, p. 657.

EXERCISE 21

Genus Pasteurella

Small rods showing bipolar staining. Gram negative. Non-motile. Aerophilic. Fermentative powers slight, no gas production. Gelatin not liquified. Parasitic, frequently highly pathogenic.

Members of this group produce plague in man, and hemorrhagic septicemia in lower animals. The organism is found in the local lesions and the blood stream of infected individuals, and in the lymph nodes of rats that carry the bacilli. *B. leipsepticus* (*Pasteurella cuniculicida*) is studied in the classroom as the representative of the group.

A. Material needed:

24 hr. cultures of *B. leipsepticus*

1 dextrose agar plate (required only if exercise is done separately)

1. Make methylene blue and Gram stained films of culture. Fix films after drying by flooding with alcohol and allowing the alcohol to burn off.

2. Inoculate a portion of an agar plate with the culture.

B. Examine culture on agar plate. Prepare films: fix with alcohol. Stain by methylene blue and toluidine blue. Examine for capsules. Examine for motility in fresh preparation.

REFERENCES:

Besemer—The *Pasteurella* Group. Jour. Bact., 2, 1917, p. 177.

Castellani and Chalmers—Manual of Tropical Medicine, W. Wood & Co., N. Y., 1919.

McCoy, G. W.—American Jour. Hygiene, March, 1921.

Genus Pfeifferella

Slender rods, sometimes forming filaments and showing a tendency toward branching. Non-motile. Gram negative; staining poor, with characteristic irregularities. Carbohydrates not fermented. Growth on potato characteristic.

The *Pfeifferella mallei*, or bacillus of glanders, is the only species of the genus. It is primarily a disease of horses, and is found in the local lesions and occasionally in the blood stream. The frequency and severity of laboratory infection by the glanders bacillus make it undesirable to use living cultures in class study. Killed suspensions may be examined for staining and morphology, which is similar to that of the *Pasteurella* group.

Bacillus Melitensis (*Bacillus Alkaligines Melitensis*)

This organism is the cause of Malta fever in human beings, who

generally contract the disease through drinking the milk of infected goats. Formerly regarded as a micrococcus, it has been shown definitely to be a rod, and is identical, in fact, with the causative organism of bovine abortion, named *B. abortus* by Bang.

The organism can be secured from milk of goats and cows, from aborted foetuses and the surrounding membranes, from the blood of patients, and the organs of victims.

Students will be given killed suspension of *B. melitensis*. On account of the frequent laboratory infections which have occurred, living cultures will not be supplied in class.

1. Note appearance of agar cultures of *B. melitensis* on demonstration table.

2. Make Gram's stain of the suspension given out.

REFERENCES:

MacNeal and Kerr—*Bacillus abortus* of Bang, the Cause of Contagious Abortion in Cattle. *Jour. Inf. Dis.*, vol. 7 (1910), p. 469.

Meyer, K. F., and Shaw, E. B.—A comparison of the morphologic, cultural, and bio-chemical characteristics of *B. abortus* and *B. melitensis*, *Jour. Inf. Dis.*, vol. 27 (1920), p. 173.

INFECTION AND IMMUNITY

20. *Human Blood Typing.* Secure blood, by pricking the finger, from members of the class. Separate erythrocytes and serum, and set up cross agglutination tests, testing each serum against each of the specimens of cells. Prepare table of results.

REFERENCES:

Landsteiner—Ueber Agglutinationserscheinungen normalen menschlichen Blutes. *Wien. klin. Woch.*, Vol. 14 (1901), p. 1132.

Moss—Studies on Iso-agglutins and iso-hemolysins. *Trans. Ass. Amer. Phys.*, vol. 24 (1909), p. 419.

Ottenberg—Blood Grouping. *Jour. Amer. Med. Assn.*, Vol. 77 (1921), p. 682.

Gay—The Functions of Tonicity in Human Isohemagglutination. *Jour. Med. Res.*, XVII, 1907, P. 321.

Landsteiner—In Jordan and Falk, chapter 68.

21. *Precipitins.*

- (a) Immunize a rabbit against human blood serum. Test the serum of the rabbit for specific precipitation, using the homologous antigen and sera of laboratory animals.

Zinsser et al., p. 94.

- (b) Demonstrate the forensic value of this antiserum in the identification of human blood stains.

Zinsser et al., p. 98.

EXERCISE 22

Family Bacillaceae

Genus Bacillus

Large rods, producing endospores. Growth vigorous on artificial media. Often occur in long chains and produce rhizoid colonies. Flagella when present peritrichous. Gram positive. Generally proteolytic.

The *Bacillus anthracis* is the pathogenic member. It is of high virulence, occurs chiefly as a disease of cattle and may be transmitted from animal sources to human beings. The bacillus is found in the local lesions, and is found at death in great numbers in the blood and organs.

The *Bacillus subtilis* is a widely distributed member of no pathogenic property for human beings, and is frequently encountered as a contaminant in cultures.

A. *Material needed:*

- 24 hr. culture of *B. subtilis*
- Guinea pig infected with *B. anthracis*
- 2 deep agar tubes
- 2 Petri plates

1. Pour plates. Make streak inoculation of loopful of blood or fluid from spleen of infected guinea pig on surface of agar plate.

2. Prepare films of blood or organ fluid and stain by Gram and by Wherry's capsule stain.

3. Make streak inoculation of *B. subtilis* on second agar plate.

B. *Material needed:*

- 2 gelatin tubes
- 1 agar slant

1. Examine colonies of *B. anthracis* and *B. subtilis* on agar plates. Make fresh and stained preparations.

2. Inoculate gelatin tube by stab culture from colony of each bacillus. Incubate at room temperature.

3. Emulsify a loopful of agar culture of *B. subtilis* in 1 c.c. of saline solution. Heat in water at 80° for 15 minutes. Subculture saline emulsion to agar slant. Incubate at 37°.

C. 1. Examine gelatin tubes for liquefaction. Examine agar slant from heated *B. subtilis* suspension for growth.

REFERENCE:

Hutyra and Marek—*Specielle Pathologie und Therapie der Haustiere*. Bd. 1, p. 25.

Dawson—*Methods of Anthrax Vaccination*. Bureau of Animal Industry, Bulletin 137, 1911.

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22. Inoculate a guinea pig subcutaneously with culture of *B. anthracis*. After death of the animal, autopsy. Make stained preparations and cultures from spleen, liver and heart's blood.

REFERENCE :

Wadsworth, p. 247.

23. *Bacterial Precipitin*. Prepare antigens from cultures of pneumococcus and *B. typhosus*. Demonstrate bacterial precipitins with the use of these antigens and the homologous antisera.

REFERENCES :

Kolmer, p. 1135.

Gay & Chickering of Exp. Med. 21, 1915, 389.

EXERCISE 23

Genus Diplococcus (Pneumococcus)

Strict parasites, nutritive requirements exacting. Fresh cultures dissolved by bile. Inulin fermented.

A. *Material needed:*

- 24 hr. cultures of pneumococcus types I and III
- Mice infected with pneumococcus
- 2 blood agar plates
- 1 infusion bouillon tube
- 1 inulin serum water tube

1. Make fresh and stained preparations of stock cultures of pneumococcus.

2. Inoculate bouillon tube and inulin serum water medium, and make streak inoculations on blood agar plate.

3. Streak out heart's blood of infected mouse on a blood agar plate.

B. *Material needed:*

- Wherry capsule stain
- Blood serum or ascitic fluid
- M
- H C1
- 20
- Wassermann tubes
- 3% washed red blood cell suspension
- Sterile bile or bile salt solution
- Water bath at 37° C.
- Diluted anti-pneumococcus sera
- 1 blood agar slant

1. Observe characteristics of cultures of stock pneumococcus on blood agar plate. Note character of pneumococcus mucosus growth. Examine by Gram stain, and by Wherry capsule stain, as follows:

Emulsify a portion of culture in a loopful of serum on a slide.

M

Mix with this an equal amount of —H C1. Allow to stand one 20

minute, then spread out in a thin film in the usual way. Allow to dry, and fix quickly with heat or by flooding with alcohol. Stain one minute with dilute gentian violet or dilute carbol fuchsin. Wash with several changes of distilled water and dry without blotting. Examine with high power dry objective, and if stain is too light, restain the slide; if the stain is too heavy, repeat the washing with distilled water. Repeat examination with high power dry lens, and examine finally with the oil-immersion lens.

2. Note character of growth in bouillon. Examine in fresh preparation. Compare with staphylococcus and streptococcus growth.

3. Set up test for hemolysis or methemoglobin formation, and for solubility of the culture by bile, as in streptococcus exercise. In addition, set up three tubes containing 10 drops of culture, and add to each 5 drops of anti-pneumococcus serum, types I, II and III. Place tubes in water bath for 30 minutes, and examine for agglutination.

4. Examine for acidification and coagulation in inulin serum water medium.

5. Examine blood agar plate from infected mouse for typical pneumococcus colonies. From isolated colony, make Gram stain, and transfer the rest of the colony to a blood agar slant.

C. Examine culture isolated from infected mouse and carry out necessary steps to complete identification as pneumococcus.

REFERENCE :

Avery, Chickering Cole & Dochez. Acute Lobar Pneumonia. Monographs, Rockefeller Inst. No. 7, Oct. 16, 1917.

Heidelberger and Avery. Soluble specific substance of pneumococci. J. Exp. Med., 38, 1923. 73, 81. J. Exp. Med., 42, 1925. 367.

Cooper, Edwards and Rosenstein. Separation of Types among Pneumococcus Group IV. J. Exp. Med., 49, 1929, 461.

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24. *Diagnosis and Differentiation of Pneumococcus Type in Sputum.* Follow Rockefeller Monograph No. 7. If Sputum from lobar pneumonia is not available, infect normal sputum with pure culture.

Wadsworth, p. 131.

25. *Protective Value and Specificity of Antipneumococcus Serum*

Type I. Carry out with mice, and control with normal horse serum and Type II pneumococcus.

Follow Rockefeller Monograph No. 7.

See also Kolmer p. 409.

EXERCISE 24

Genus Encapsulatus

Plump rods with rounded ends. Short, almost coccoid, or long forms. Occur singly, in pairs, or short chains. Non-motile. Encapsulated. Gram negative. Abundant growth and active fermentation of carbohydrates.

The *Bacillus mucosus capsulatus*, or Friedländer bacillus, is the important member. It is found as a pathogen in infections of the upper respiratory tract and less commonly in lobar pneumonia and other infections.

A. *Material needed:*

- 24 hr. culture of *Encapsulatus pneumoniae* (*B. mucosus capsulatus*.)
- 1 deep tube of dextrose agar
- 1 Petri plate
- Dextrose fermentation tube
- Lactose " "
- Saccharose " "

1. Pour agar plate. Make streak inoculation of the culture on a portion of the surface. Reserve the remainder of the surface for another organism.

2. Examine stained and fresh preparations of the culture.

3. Inoculate the fermentation media.

B. *Material needed:*

- Blood serum or ascitic fluid
- N
- HCl
- 20

1. Observe characteristic growth on agar plate. Note and record fermentation reactions.

2. Make fresh preparation from growth in fluid medium and examine for motility.

3. Stain a portion of growth on agar for capsules as in previous exercise (Exercise 24).

REFERENCES:

- Fitzgerald—A Biometrical Study of the Mucosus Capsulatus Group. Jour. Inf. Dis., 15, 1914, p. 268.
- Coulter—Biological Identity of the Friedländer Bacillus. Jour. Exp. Med., 26, 1917, p. 763.
- Julianelle—A Biological Classification of *Encapsulatus Pneumoniae*. Jour. Exp. Med., 44, 1926, p. 113.

EXERCISE 25

Genus Hemophilus

Minute rod-shaped cells, often thread forming and pleomorphic. Non-motile. Strict parasites growing best, or only, in the presence of hemoglobin and in general requiring blood serum, ascitic fluid or other source of growth accessory substances. Gram negative.

B. (*Hemophilus*) *influenzae* is the most common member. Etiological studies in recent epidemics of influenza have brought into serious question the etiological relation of this organism to the epidemic disease. The bacillus is found in sputum from the lung, often in association with a streptococcus in certain forms of pneumonia, and occasionally causes meningitis in infants. The bacillus occurs also in the nasopharynx of patients and carriers.

The bacillus *pertussis* of Bordet is culturally similar, but immunologically represents a distinct species. It is to be found in the sputum from cases of pertussis. A special medium is necessary for its primary isolation.

The Unna-Ducrey bacillus of chancroid is found in the pus in chancroidal ulcer or in pus aspirated from a bubo. The organism is difficult to cultivate and grows best on whole coagulated rabbit blood which has been heated to destroy alexin.

A. *Material needed:*

- 24 hr. cultures of *B. influenzae*
- B. pertussis*
- 2 deep tubes of agar
- 2 c.c. sterile defibrinated blood
- 2 c.c. sterile laked blood
- 2 Petri plates

1. Pour blood-agar plates of defibrinated blood and laked blood. Allow to harden. Divide each plate into halves and inoculate each half by streaking with each of the cultures supplied. Incubate at 37°.

2. Examine stained films of cultures, using Gram stain with diluted (1/5) carbol fuchsin as the counterstain.

3. Stain films of sputum showing *B. influenzae*, if available.

B. Study colonies on blood-agar plates. Make stained films from each culture.

REFERENCES:

- Bordet-Gengou—Collected Studies, p. 472.
- Wollstein, M.—*Jour. Exp. Med.*, XI, 1909.
- Avery—*Jour. A.M.A.*, vol. 71, 1918, p. 2050.
- Thjotta and Avery—Studies on Bacterial Nutrition. *Jour. Exp. Med.*, XXXIV, 1921, p. 97.

- Povitsky—Improved Methods for the Isolation of *B. Pertussis*. Jour. Inf. Dis., 32, 1923, p. 8.
Krumwiede, Mishulow and Oldenbusch—Types of *B. Pertussis*. Jour. Inf. Dis., 32, 1923, p. 22.

INFECTION AND IMMUNITY

26. *Fixation of Alexin.*

See Bordet-Gay, p. 189.

Plan and demonstrate a similar experiment, with materials approved by the instructor.

Zinsser et al., p. 100.

EXERCISE 26

Isolation of Pathogenic Bacteria from Naso-Pharynx

Members of the three groups which have just been studied are often found in the nasopharynx of convalescent patients and healthy carriers. Isolation of the organism is often attempted in order to detect such carriers. Cultures are taken from the mucous membrane of the nasopharynx with a small cotton swab on the end of a straight or bent wire and planted on various media. Blood-agar and laked blood agar plates are the most useful media. Primary cultivation in a fluid medium is often used in the isolation of streptococcus or pneumococcus when present in small numbers. Spraying the mucous membrane with a mild antiseptic an hour before the culture is taken has been used with success to kill the saprophytic bacteria present on the free surfaces and permit recovery of the pathogenic forms lodged and multiplying in crypts and folds of the mucous membranes.

A. *Material needed:*

- 2 sterile wire swabs
- 1 deep tube of dextrose agar
- 2 c.c. sterile laked blood
- 1 Petri plate
- 1 tube infusion bouillon

1. Take a culture from the retropharynx of your neighbor. Pass the sterile wire swab back of the soft palate, with care not to touch the mucous surface of the mouth, and gently wipe the mucous membrane of the retropharynx, as high above the level of the soft palate as possible, with the swab. Withdraw the swab carefully.

2. Make a single streak with the swab across the surface of a laked-blood agar plate. Streak this portion out further with the loop, passing at right angles to the first streak. It requires care and experience to make the plate inoculation sufficiently light.

3. Emulsify the material remaining on the swab in a tube of infusion broth.

Discard the swab.

4. Incubate cultures at 37°.

B. *Material needed:*

2 blood agar slants

1 blood agar plate

1. Examine the laked blood agar plate for colonies of meningococcus or *B. influenzae*, using the hand lens, and making stained films from marked colonies. Subculture suspicious colonies (2) to blood agar slants. Incubate at 37°.

2. Do not agitate bouillon culture. With a capillary pipette examine (a) the body of the liquid and (b) the sediment, in fresh preparations. If chain-cocci are found, place a drop of the culture liquid on the surface of a blood agar plate, and streak out over the plate with the loop. Incubate at 37°.

C *Material needed:*

Normal, antimeningococcus and anti pneumococcus horse serum

Wassermann tubes

Water bath at 55°.

1. Examine growth on blood agar slants and make stained films. If an organism resembling the meningococcus is found, emulsify growth in saline and set up agglutination test against normal and anti-meningococcus horse serum.

2. Examine blood agar plate made from bouillon culture for colonies of pneumococcus or streptococcus. The hemolytic streptococcus is easily identified by the zone of hemolysis. Subculture suspected colonies of either form to infusion bouillon and after incubation determine purity of culture and set up tube-test for hemolysis, bile solubility and agglutination by antipneumococcus sera.

EXERCISE 27

Genus Corynebacterium (Diphtheria Bacillus Group)

Slender rods, with characteristic morphology; often curved, pointed or club shaped, with occasional branching forms. Irregular barred staining. Gram positive. Some species produce exotoxin.

The diphtheria bacillus is the most important and perhaps the sole pathogenic member. It is found in the characteristic necrotic lesions on mucous surfaces, and in the nasopharynx of carriers.

A. *Material needed:*

24 hr. cultures of *B. diphtheriae*
 B. xerosis
 B. hoffmani

3 Loeffler's serum tubes
3 dextrose serum agar slants
3 saccharose serum agar slants

1. Prepare films of stock culture. Stain with methylene blue, toluidine blue and Neisser stain (acid methylene blue, followed by Bismarck brown). Observe pleomorphism. Examine for characteristic forms, for polar bodies. Differentiate *B. diphtheriae* from other members of the group.

2. Stain films by Gram.

3. Inoculate Loeffler's serum medium and fermentation agar slants with stock cultures.

B. *Material needed:*

Field throat cultures, "positive" and "negative"
1 dextrose agar plate
1 ascites agar plate

1. Make preparations from Loeffler's serum cultures of members of this group and stain as in previous exercise.

2. Note fermentation reactions on dextrose and saccharose media.

3. Emulsify a loopful of growth of each member of group in 2 c.c. of saline solution and streak out on dextrose agar plate.

4. Prepare films from field throat cultures. Stain as directed in A.1 Examine for presence of *B. diphtheriae*.

5. Emulsify one loopful of growth from throat culture containing suspected *B. diphtheriae* in 2 c.c. of saline solution and streak out on ascites agar plate. Incubate at 37° C.

C. *Material needed:*

2 Loeffler's serum tubes

1. Study colonies of pure cultures on dextrose agar plates. Examine with hand lens and low power microscope. Fish from isolated colonies and make stained preparations, using methylene blue.

2. Study ascites agar plate made from positive throat culture. Search for characteristic colonies of *B. diphtheriae*; fish from these, make stained preparations and if characteristic morphology is observed, inoculate Loeffler's serum tubes. After growth, complete necessary steps to make cultural identification of *B. diphtheriae*.

REFERENCES:

Nuttall and Grahām-Smith—The Bacteriology of Diphtheria. Cambridge University Press, 1908.

Mellon, R.—A Study of the Diphtheroid Group of Organisms. Jour. Bact., 2, 1917, p. 81, 269, 447.

Park, Williams and Mann—Types of Diphtheria Bacilli. Jour. Immunology, 7, 1922, p. 243.

INFECTION AND IMMUNITY.

27. *Schick Test*. Secure specimen of standardized diphtheriae toxin. Make test by intracutaneous inoculation, on students who volunteer, for presence of diphtheriae antitoxin in circulating blood.

REFERENCES:

Kolmer, p. 739.

Park and Williams.

29. *Virulence Determination of Field Cultures*. Obtain a field diphtheria toxin and with mixture of toxin and antitoxin. Use three guinea pigs in each series and make approximate determination of the MLD and L+ values for the toxin and antitoxin employed.

REFERENCES:

Zinsser et al.

Standardization of Diphtheria Antitoxin, Hyg. Lab. Bulletin, No. 21, 1905.

Park and Williams.

29. *Virulence Determination of Field Cultures*. Obtain a field culture containing a suspected toxin-producing diphtheriae bacillus. After incubation over-night, emulsify culture in saline, and inoculate intracutaneously into two guinea pigs one of which has previously received diphtheria antitoxin. Examine for development of local necrotic lesion in the unprotected pig.

REFERENCES:

Wadsworth, p. 148.

Park and Williams.

EXERCISE 28

Genus Mycobacterium (Acid Fast Group)

Slender rods, frequently irregular in form, occasionally branched. Cells stain with difficulty; when once stained, are acid-fast. Young forms may not retain stain. Parasites. Growth slow, cultural requirements exacting.

The types of *B. tuberculosis* and *B. leprae* are the pathogenic members. The organisms are found only in the lesions of disease, or in discharges, such as sputum where they may remain alive for considerable periods of time.

A. *Material needed:*

- Cultures of *B. tuberculosis*
 - B. smegmatis*
 - B. phlei* (timothy-hay bacillus)
- Tuberculosis sputum or pus
- Guinea pig infected with *B. tuberculosis*
- 3 glycerine agar slants
- 1 Dorset egg medium tube.

1. Prepare films from stock cultures and stain by Ziehl-Neelson stain. Observe morphology and staining reaction.

2. Inoculate glycerine agar slants from stock cultures. Incubate at 37° in small incubator. The cultures should be kept for two weeks or longer.

3. Prepare films of tuberculous material, and stain by Ziehl-Neelson. Always dry the loop at the side of the flame, before complete flaming, after taking up material from tuberculous processes.

4. Inoculate material from closed lesion in guinea pig upon Dorset egg medium. Close tube with paraffined cork. Incubate with other cultures.

B. Examine cultures made from stock strains and from lesion in infected animal, when growth occurs. Make fresh preparations and films which should be stained by Gram and by Ziehl-Neelson stains.

REFERENCES:

- Park and Krumwiede—Bovine and Human Types in Different Forms of Tuberculosis. Jour. Med. Res., 27, 1912, p. 109.
- Calmette, A.—L'Infection Bacillaire et la Tuberculose. Masson et Cie, Paris. Also in English translation, Soper and Smith: Tubercle Bacillus Infection and Tuberculosis in Man and Animals. Williams & Wilkins, Baltimore.
- Krause, A. K.—Experimental Studies in Tuberculous Infections. Am. Rev. of Tuberculosis, Vol. VI, No. 1, 1922.

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30. Inoculate guinea pigs by subcutaneous injection with sputum containing tubercle bacilli. Follow course of activity and weight of the animals. Three weeks after the inoculation give to the inoculated animals and a control 0.5 c.c. Koch's O. T. subcutaneously. Autopsy animals, examine lesions in the gross and in microscopic section. Prepare films from the lesions and stain by Ziehl-Neelson.

REFERENCES :

Wadsworth.
Kolmer

31. Using the cutaneous or intracutaneous method of inoculation, test members of the class who volunteer for reactivity to tuberculin. Kolmer, p. 689.

EXERCISE 29

Genus Clostridium (Group of Sporulating Obligate Anaerobes)

Anaerobes or microaerophiles. Rods, frequently enlarged at sporulation, producing clostridium or plectridium forms. Young individuals Gram positive. Two groups; saccharolytic and proteolytic. Often parasitic.

Anaerobes are found abundantly in soil, water, the lower intestine, and putrefactive processes generally. The proteolytic group includes the important organisms of anaerobic putrefaction. The anaerobes occur frequently in diseased processes, for the most part of low virulence, but may possess high independent pathogenicity. Material from wound infections and gaseous gangrene provides the best source. Primary cultures are usually mixed and contain both anaerobes and aerobes.

A. *Material needed:*

- cultures of *B. welchi*
 - B. sporogenes*
 - B. oedematiens*

Plate cultures of above, *B. tetani* and *B. botulinus* for demonstration

- 1 agar slant
- 1 deep dextrose agar tube
- 1 milk tube
- 1 chopped meat bouillon

1. Observe colony characteristics of anaerobic bacilli on demonstration plates.

2. Make fresh and stained preparations from stock cultures. To examine for motility, make slide cover-slip preparation, and rim the cover-slip with vaseline to exclude air from the film of liquid.

3. Each student will inoculate the media supplied with one of the stock cultures. Place the tubes of agar, milk and chopped meat bouillon in a cup filled with water and heat to boiling for 15 minutes. Cool media quickly. Inoculate the tube of agar at 45° C., distribute inoculum uniformly, and cool by placing tube in cold water. After inoculation cover the surface of the milk and chopped meat bouillon with a layer of sterile melted vaseline. Inoculate the agar slant to test for aerobic contamination.

B. 1. Examine subcultures during incubation for 2 to 14 days. Examine for turbidity, gas formation, coagulation of milk, and digestion of milk coagulum and meat substance.

2. Make fresh and stained preparations using Gram stain.

3. Observe characteristics of colonies visible in deep agar tube; compare with similar cultures of other varieties. Use a very fine capillary pipette to take up a colony or portion of one for microscopic examination.

REFERENCES :

Hall—Differentiation and Identification of the Sporulating Anaerobes. Jour. Inf. Dis., vol. 30 (1922), p. 445.

Dickson—Botulism Rockefeller Inst. Med. Res., Monograph No. 8.

Weinberg et Seguin-La Gangrene Gazeuse, Masson, Paris, 1918.

Medical Research Committee—Reports of the Committee upon Bacteria and Infection. London, 1919.

INFECTION AND IMMUNITY

32. Prepare 24 hr. cultures of *B. welchi*, *B. histolyticus*, *B. oedematiens*, *B. sporogenes*, and *Vibrio septique* in chopped meat bouillon. Inoculate guinea pigs intramuscularly. Observe development of lesions. Autopsy: make stained films and cultures from lesions and from heart's blood, and examine for the presence of the anaerobes.

Kolmer, p. 871.

33. *Pathogenic Action of B. tetani*. Insert splinter the tip of which has been dipped into a culture of *B. tetani* through skin incision into thigh muscle of guinea pig. Observe development of symptoms. Autopsy. Examine for lesion in region of the splinter. Make anaerobic cultures from this region and from heart's blood and examine for *B. tetani*.

Kolmer.

34. *Tetanus Toxin and Antitoxin*.

Inject two guinea pigs intramuscularly with like amounts of tetanus toxin. As soon as local symptoms are evident, give one animal tetanus antitoxin intravenously. Keep the other pig as a control. Inoculate a third pig with an overneutralized mixture of toxin-antitoxin containing the same amount of toxin as given to the first two animals. Observe over a period of six days.

Kolmer, p. 1113, 1125, 933.

Rosenau & Anderson—Standardization of Tetanus Toxin, Bull. Hyg. Lab. No. 43, 1908.

EXERCISE 30

Pathogenic Fungi

The fungi are Thallophyta entirely devoid of chlorophyll. They possess in the great majority of cases a vegetative portion or mycelium made up of colorless filaments or hyphae, and reproduce by spores which may be sexual in origin.

The fungi are the causative agents of many diseases of plants. A few forms produce disease in animals. The fungi are very important in industrial bacteriology because of their highly developed fermentative properties.

The student should consult the text books particularly Zinsser for the description and the nomenclature of the morphology and the complicated reproductive structures, on which the classification of this group is based. The main classes are:

Phycomycetes: fungi in which the mycelium is usually without septa. Reproduction takes place sexually by fusion of undifferentiated or well differentiated male and female cells or asexually by the formation of conidia. A common example is *Mucor mucedo*, a black mold which occurs frequently as a laboratory contaminant.

Ascomycetes: in this group belong the yeasts, many of the common molds of which *Aspergillus* and *Penicillium* are examples, and a number of important plant parasites including ergot (*Claviceps purpurea*).

Basidiomycetes: in this class belong the mushrooms and similar fungi, the rusts, parasitic on leaves of wheat and barberry, and the smuts, a common variety of which infects ears of corn. None of this class have been proved to cause disease in man.

Fungi Imperfecti: this group is an artificial one in which are placed for convenience forms whose sexual reproduction has never been observed. Only the conidial stage is known. In this group belong all the fungi parasitic for man.

The Moniliae and Blastomycetes are closely related to the yeasts. The *Sporothrix* and Ringworm fungi also are similar to certain ascomycetes except in their lack of ascospores. The actinomycetes are placed in this group by some and by others are considered as types of higher bacteria.

Examples of forms associated with human disease are the following:

Aspergillus. A number of species of this genus are frequently annoying contaminants in bacterial cultures. A black species, *Aspergillus niger* is found in inflammations of the external auditory canal and a dark green or grayish species, the *Aspergillus fumigatus*,

has been found in the lung in cases of chronic infection resembling tuberculosis. Their etiological relationship to the lesions has not been absolutely proven.

Microsporon lanosum: This organism is frequently the cause of ringworm of the scalp in children. In the infected hairs it develops a delicate mycelium and forms great numbers of minute round spore-like bodies which form a thick sheath surrounding the hair immediately beneath the cuticle.

Trichophyton Gypseum: This is a common cause of suppurating ringworm of the scalp and beard and of ringworm of the palms and soles. As seen in the hairs it resembles *microsporon lanosum* but the sheath of the spores is less well developed. In the skin covering vesicles on the palms and soles, and in the squames from scaling lesions, it is seen as a network of septate branching and anastomosing filaments. The colonies on agar form a tough felt and develop into huge and nearly flat colonies.

Actinomyces: This group represents the so-called higher bacteria, which are believed to be intermediate between bacteria and molds. The organisms are found in nature, and in disease, causing various forms of granulomatous infection, and chronic suppurative processes. The most important type of organism is *Actinomyces Bovis*, found in the pus of a specific chronic disease of man and cattle. The actinomyces are seen in small irregular masses or colonies (Drusen) lying in granulation tissue. The center of the masses stain a pale blue and the network of delicate filaments of which it is made up cannot be discerned. In sections about the edge the tips of these filaments project in a fringe and the tip of each filament is surrounded by a hyalin sheath (club) which stains faintly with eosin.

Blastomyces: These organisms produce suppurative processes in the skin and internal organs. In sections of the lesions stained with hematoxylin and eosin, the blastomyces are seen as pale blue spherical cells surrounded by an unstained halo or capsule. They occur among collections of leucocytes and also within giant cells. Budding forms can occasionally be seen. The related form, *coccidioides immitis* as seen in tissues resembles the blastomyces. No buds are seen but some of the larger cells are crowded with minute round endospores.

Monilia psilosis: This organism occurs in the alimentary tract of patients with sprue, but is morphologically indistinguishable from the organism found in thrush and from certain other forms. It grows on agar in pasty colonies resembling giant colonies of staphylococcus. Young colonies are made up entirely of large oval cells

from the tips or sides of which smaller similar cells bud out. In older cultures the cells elongate and remain attached at their tips forming a rudimentary mycelium. Buds develop from the sides of these hyphae. Below the surface of the medium, in gelatin, for example, they form huge characteristic balls of spores surrounding the hyphae. Fusion of cells and the development of ascospores within the cells, which occurs in the true yeast, has never been observed in this species.

Materials:

- Cultures in 6% honey agar of pH 5.5
 - of *Monilia psilosis*
 - Aspergillus*
 - Microsporon lanosum*
 - Trichophyton Gypseum*
- Demonstration slides of
 - Actinomycosis*
 - Blastomycosis*
 - Coccidioidal granuloma

Technic.

Direct examination for fungi in hair or in scrapings from the skin is best made by placing a small amount of material on a slide in 10% to 30% NaOH, pressing a cover slip over it, and warming gently over a Bunsen flame. The alkali clears the horn cells and renders the fungus visible.

Cultures are made on peptone agar with a reaction of about pH 5.5 to which 4% of dextrose is added. It is better to add the dextrose in the form of crude malt or honey as the impurities present in these substances seem to favor growth. Most fungi develop poorly at 37 degrees; well at room temperature. Nutrient media of pH 7 or over, favorable for bacteria, are unfavorable for the growth of fungi.

The best morphological preparations are obtained by growing the fungus in a hanging drop of suitable broth sealed over a hollow ground slide. Satisfactory preparations also may be made by gently tearing a small fragment from an agar culture with a stiff needle, and mounting on a slide with a drop of dilute alcohol to which NaOH is later added. A cover slip is placed over the preparation which is studied unstained with the diaphragm nearly closed. Dalmau has devised a simple method for study of monilia and mold like forms. The cultures are grown in agar in Petri dishes and a cover slip gently pressed down on the area to be observed.

REFERENCES:

- Brumpt.—*Precis de Parasitologie*. Paris, Masson, 1921.
- Chalmers, A. J. & Castellani, A.—*Manual of Tropical Medicine*, New York, 1919.
- Buchanan, E. D. and R. E.—*Bacteriology*, New York, 1925. Chapter XLIII and Appendix. (Key to the Families and Genera of the commoner mold)
- Zinsser, H.—*Text Book of Bacteriology*, Chapters 49 and 50. New York, 1928.

INFECTION AND IMMUNITY

35. *Active anaphylaxis.*

Prepare vegetable globulin antigens. Sensitize guinea pigs, following the directions of Zinsser et al. The shocking dose of antigen is to be given three weeks later.

36. *Desensitization or antianaphylaxis.*

Sensitize guinea pigs as for Problem 32. Desensitize the animals at the end of three weeks according to the directions of Zinsser et al. Twenty-four hours later administer shocking dose of antigen as in 32.

REFERENCES :

Zinsser, Hopkins and Ottenberg.
Kolmer.

37. *Allergy.*

Perform skin tests with extracts of pollens, food, etc., on members of the class who have a history of hay fever or asthma. Follow the technique carried out in the allergy clinic.

Duke, W. W.—Hay Fever, Urticaria and allied Manifestations of Allergy.
C. V. Mosby Co., St. Louis, 1925.

EXERCISE 31

Spirochaetes (Spirochaetales)

The taxonomic relations of the spirochaetes are as yet unsettled. They are regarded by certain authorities as intermediate between bacteria and protozoa, being related on the one hand to the spirilla and vibrios, and on the other to the trypanosomes. The spirochaetes are widely distributed both as saprophytes and as parasites.

For the best classification which has been proposed to date the student is referred to Noguchi, J. Exp. Med., vol. 27, pp. 575-592, 1918. The genera listed are as follows:

1. SPIROCHAETA. Example, *Spirochaeta Plicatilis* living free in water.
2. SAPROSIRA. Example, *Saprosira Grandis* living free in certain sands.
3. CRISTISPIRA. Example, *Cristispira Balbiani* found in the alimentary canal of certain shell fish.
4. SPIRONEMA. Example, *Spironema Duttoni*, of African relapsing fever.
5. TREPONEMA. Example, *Treponema Pallidum*, of syphilis.
6. LEPTOSPIRA. Example, *Leptospira Icterohemorrhagica* of trench jaundice or infectious jaundice.

Cultivation of these microorganisms is most difficult and is far beyond the ability of the beginner. The student is referred to Noguchi, Journal of Experimental Medicine, vol. 30 (1919) p. 13, for a discussion of some of the problems involved.

The most suitable materials for the study of the important genus of *Treponema* are: material about the teeth, the exudate of gingivitis, blood and tissue fluids from experimentally infected animals and the serous exudate from syphilitic ulcers.

With a sterile tooth-pick obtain material from about the teeth. Spread in a thin film on a slide and stain by Giemsa's stain, as follows:

- (a) Fix film with methyl alcohol, 1 to 5 minutes.
- (b) Dilute stock Giemsa stain, 1 c.c. to 10 to 15 c.c. of freshly distilled water to which 2 drops of 1% K_2CO_3 solution are added.
- (c) Flood slide and stain 15 to 30 minutes. Renew stain two or three times during this period.
- (d) Wash with distilled water until film has slight pinkish tinge.

Serous fluid from human lesions is obtained by scraping the margin of the lesion with a sterile scalpel, taking great care to avoid the appearance of blood. In a few moments the clear serous fluid

which appears may be taken up with the point of a clean sterile scalpel or a wire loop cooled after flaming, and placed on a slide.

This exudate or tissue fluid from experimental lesions (rabbit testis) may be spread into a film, and stained by Giemsa or by the Fontana stain given below. It may also be examined by the India ink method or covered with a slip and observed directly with the aid of dark-field condenser.

The India ink method and the Fontana stain are as follows:

Burri's India Ink method:

Place a drop of India ink and a drop of spirochaeta suspension on a slide. Mix the two and spread out in a thin film by means of the end of another slide as in making a blood film. Ordinary drawing ink may be used and will probably need no dilution. It is not as satisfactory, however, as the Austrian Chin-Chin brand, which is the best, if possible to procure. Inasmuch as spirochetes may be present normally in some India inks, it is wise to make a control test with no stainable material added.

Fontana Stain:

- (a) Flood slide with Fontana I solution (Formalin 2 c.c. acetic acid 1 c.c., distilled water 100 c.c.) after 1 minute wash.
- (b) Add Fontana II (Phenol 86% 1 c.c. tannic acid 5 grams, distilled water 83 c.c.) and steam 30 seconds.
- (c) Cover preparation with Fontana III (silver nitrate 0.25 grams, distilled water 100 c.c. ammonia q.s.) Add ammonia drop by drop until the precipitate which first appears goes into solution.

The use of the dark-field will be demonstrated.

38. *The Wasserman Reaction for Syphilis.* Assemble materials for the alexin fixation test and secure Wasserman "antigen." Prepare protocols for the preliminary titration of the hemolytic system. After this has been carried out, set up the test with known sera obtained from the diagnostic department.

Kolmer
Zinsser et. al.

PROTOZOA

The majority of the protozoa are single celled organisms with the animal type of metabolism. They do not form a clearly limited group, but merge on the one hand into forms having plant metabolism and on the other into the simpler forms of metazoa, or multicellular organisms. The protozoa are subdivided into four large groups and each group contains parasitic or disease causing forms, although the majority are free living. In the following classification an example is given in each group of a parasitic form.

1. *Sarcodina*. These are characterized by the use of pseudopodia as organs of locomotion and for ingestion of food. Example: *Amoeba histolytica*, the cause of Amebic dysentery.

2. *Mastigophora*. These move by whip-like structures, or flagella. Example: *Trypanosoma gambiense*, the cause of African sleeping sickness.

3. *Sporozoa*. All of the organisms in this group are parasitic. Many of them have complicated and specialized life cycles. Example: *Plasmodium vivax*, the causative agent in one type of human malaria.

4. *Infusoria*. These animals move by means of cilia. This group contains the most complex and highly developed protozoa. Few of them are parasitic. Example: *Balantidium coli*, the cause of a relatively rare disease characterized by ulceration of the large intestine.

The majority of the protozoa can be cultivated only with the greatest difficulty or not at all. It is thus necessary to use animals as reservoirs of the parasitic forms. Hence the work in protozoology in this course will consist of problem demonstrations, and demonstrations of prepared slides, since the number of animals otherwise required would be too great to be practicable.

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39. *Trypanosome infections*. A comparison between a fatal and a non-fatal trypanosome infection.

1. Infect a rat with *Trypanosoma equiperdum* by injecting it intraperitoneally with blood from an infected guinea pig. Make daily blood films to determine the first appearance of the parasites in the blood. After they have entered the blood stream, make daily total counts of the parasites until the time of death. Count also the white blood cells.

2. Infect a second rat as before, but use twice as much guinea pig blood and study it in the same way. Is the incubation time in the rat affected? Is the survival time of the rat changed?

3. Infect a rat with *Trypanosoma lewisi*. Follow the course of the infection as before, using both blood films and total counts. Pay particular attention to morphological changes in the parasites.

4. Infect a second rat with twice the amount of blood and study as before.

Graph all results.

References: Hegner and Taliaferro.—Human Protozoology, N. Y., 1924, pp. 133-143.

Taliaferro, W. H. & L. G.—The Resistance of Different Hosts to Experimental Trypanosome Infections. Amer. Jour. Hyg. 1922, 2:264-319.

Taliaferro, W. H.—Reaction Products in Infections with *T. lewisi* which Inhibit the Reproduction of the Trypanosome. Jour. Exp. Med. 1924, 39:171-190.

40. Infection with *Bartonella Muris* (Pernicious anaemia of rats). Count the red and white cells of six white rats and make a differential count of the white cells with Wright stain. Splenectomize these rats. Make determinations as above on the following days, until death or recovery has taken place. Also make stain with Giemsa's Solution to show the parasites. Graph the results.

Reference: Ford, W. W. & Eliot, C. P., Jour. Exp. Med. 1928, 48:475-492.

41. *Bird Malaria*. A Study of the life cycle of *Plasmodium praecox* in the canary.

Inject a canary into the breast muscle with a small quantity of blood from a chronically infected bird. The method of obtaining the blood will be demonstrated. Observe the canary until it appears sick, then withdraw a drop of blood, spread and stain with Wright.

Note the various forms of parasites present. Make similar stained films at various periods until a complete cycle has been obtained. Blood should be taken not oftener than once in 36 hours.

REFERENCE:

Taliaferro, W. H. Host Resistance and Types of Infection in Trypanosomes and Malaria. Quart. Rev. Biol. 1926, 1:246-269.

Taliaferro, L. G. Infection and Resistance in Bird Malaria. Amer. Jour. Hyg. 1925, 5:742-789.

FILTERABLE VIRUSES

Certainly to be classified among the external animate agents of disease are the "filterable viruses" whose existence was suspected in the earliest days of bacteriology and demonstrated as early as 1899. These agents differ from bacteria and animal parasites in that they are too small to be seen with our present methods, and they pass through porcelain filters that retain bacteria. They are responsible for over sixty different diseases of man, mammals, fish, birds, insects, plants, and probably bacteria. They cannot as yet be grown in pure culture in the bacteriological sense, and probably do not multiply elsewhere than in the living cells that they specifically affect. The affected tissue of an animal in the course of the disease is the only material so far available for the study of the agent and transmission of the malady.

The changes which these viruses produce in the susceptible cells are specific, and the "inclusion bodies" there present represent either a life form of the virus itself or a specific degeneration product due to it. By comparative methods these viruses are estimated to be about five times as large as protein molecules. Another point of interest and importance is the intense and durable active immunity that may follow either recovery from a virus disease, or artificial immunization against it.

Owing to the difficulties in studying this group of diseases it is only in very recent years that they have occupied the attention of more than a handful of investigators. It is not yet possible for beginners to study them first hand owing to the inherent difficulties and the expense of the living material involved.

Demonstrations of certain of the filterable virus diseases and of methods of their study will be given.

1. METHODS OF FILTRATION

The ability of virus to pass through the pores of a filter is dependent upon a number of factors such as size of infective particles, the electrical charges of virus and filter, the duration of filtration, degree of pressure employed, temperature at which filtration is conducted, and presence of proteins or other substances in the virus-extract that may adsorb the active agent.

Various types of siliceous filters commonly used for filtration studies will be shown.

Suspensions of a basic and an acidic dye will be passed through a Berkefeld candle to demonstrate the adsorption and retention by the filter of positively charged particles.

2. THE BACTERIOPHAGE PHENOMENON

(*Twort-d'Herelle*)

There is dispute as to whether this phenomenon of lysis of bacteria by filtered products of certain varieties of the same micro-organism is due to an extraneous living agent (d'Herelle) or some inheritable metabolic disorder. (Bordet). The phenomenon will be demonstrated.

3. MOSAIC DISEASE OF TOBACCO

Mosaic disease of tobacco is of great economic importance since it occurs among tomatoes, peppers, and potatoes as well as tobacco, resulting in a marked reduction in crop yield.

A culture of *B. pyocyaneus* or *B. prodigiosus* will be added to virus-sap extracted from mosaic diseased tobacco plants and the mixture filtered through a sterile Berkefeld filter. A portion of the filtrate will be tested on media for the presence or absence of the control organism. The filtrate will also be inoculated into healthy tobacco plants to determine the presence or absence of virus.

Inclusion bodies characteristically present in the cells of infected tissues will be demonstrated as well as plants previously infected with the mosaic disease.

The demonstration of a specific immune reaction to mosaic disease will also be given.

4. THE VIRUS OF RABIES (*Hydrophobia*)

The virus of rabies is a neurotropic virus,—that is, it apparently grows only in the tissues of the nervous system, especially the central nervous system. It is excreted, for the most part, through the saliva. It is filterable through some bacterial filters, though with difficulty.

Rabies is a disease chiefly of dogs, and spread from dog to dog, to other animals, or to man, and almost exclusively by biting.

The virus, as occurring in dogs, and as passed from dog to dog, is known as STREET virus. If this is injected into a rabbit, intracerebrally or subdurally, the rabbit will come down with rabies. If some of the brain or cord tissue of the latter rabbit is injected into yet another rabbit, and so forth for a considerable number of passages, the virus will become modified so that it will be much more virulent for rabbits, but less virulent for dogs, and almost or entirely avirulent for humans. It is now called FIXED virus, and it is

from fixed virus, either more or less virulent, or entirely avirulent, that the various antirabic vaccines are made.

Stained Negri Bodies will be demonstrated. These are cell inclusion bodies which are pathognomonic for rabies. They are considered by some to be a visible form of the rabies parasite, and by others to be a reaction by certain of the more resistant types of nerve cells to the invasion of the rabies organism. Negri bodies are hard to find in fixed virus infections, and some authorities state that they are always absent in this condition.

A rabbit coming down with fixed virus rabies will be demonstrated.

A rabbit will be infected with rabies by an injection of fixed virus into the fourth ventricle.

A rabbit fixed virus cord, attenuated by the classical Pasteur method of drying, will be demonstrated.

5. POLIOMYELITIS

Infantile Paralysis (Acute Anterior Poliomyelitis) is an acute febrile disease producing at first symptoms of a systemic infection and later flaccid paralysis of muscle groups. The disease was transmitted to monkeys by Landsteiner and Popper in 1909 by intraperitoneal injection of spinal cord from fatal cases. The disease has not been transmitted to any other animals in spite of repeated efforts. The symptoms and pathology in monkeys are very similar to those in humans.

The virus of poliomyelitis is easily filterable through Berkefeld filters. It resists glycerine, cold and drying over long periods of time.

No intracellular inclusions have been shown to occur in this disease. The microscopic pathology of the spinal cord is entirely typical—consisting of perivascular infiltration with small round cells and lysis of the motor cells of the anterior horn with neuronophagy of these degenerated cells.

Recovery from the disease leaves an immunity with virucidal antibodies in the blood. Convalescent serum has been used in treatment with apparently beneficial results if used before paralysis sets in.

An acute case of poliomyelitis in a monkey will be shown.

Recovered cases in monkeys will be shown if available.

Microscopic demonstration of cord from paralyzed monkeys.

6. HERPES VIRUS

Herpes simplex, commonly known as cold sores or fever blisters, contain a virus which is analogous to other so-called filterable agents. Immunologically, it also resembles other filterable agents in producing an active immunity. Animals that have been immunized possess substances in their blood which completely neutralizes the virus.

Lethargic Encephalitis—The cause of lethargic encephalitis in man is not known with certainty. According to Levaditi it is an auto-sterilizable infection due to an adaptation of the virus of herpes to the central nervous system. At all events a form of encephalitis somewhat resembling the human disease may be produced in rabbits and guinea pigs by herpetic material from man.

Skin lesions will be demonstrated on a guinea pig infected with a weak strain of herpes virus.

A rabbit will be inoculated intracerebrally with a brain emulsion from a rabbit dead of herpetic encephalitis. Symptoms and autopsy findings will be demonstrated.

7. VACCINIA

Cowpox or vaccinia had long been connected in the popular mind with smallpox (variola) before Jenner in 1798 showed that persons who had recovered from cowpox (contracted while milking) were immune to smallpox. Vaccinia in human beings is a localized papulo-vesicular lesion giving rise to only a mild general reaction. It can be transmitted to rabbits, guinea pigs, fowls and some cold blooded animals. The virus grows profusely in the rabbit's testicle. Variola can be transformed into vaccinia by passage through a calf or rabbit proving that vaccinia virus is a modified form of variola; the reverse transformation has not so far been accomplished.

The virus of vaccinia is filterable with some difficulty; it resists glycerine and is extremely susceptible to heat. It can be separated from the suspension fluid in the form of fine granules by differential centrifugalization. Inoculated on to the rabbit's cornea it produces typical cytoplasmic inclusion bodies.

Immunity to vaccinia is associated with so-called viruscidal antibodies in the blood, but appears before and persists longer than these antibodies. Vaccination of an immune individual produces an inflamed indurated papule (allergic reaction), at the site in 24-48 hours: vaccination of a partially immune individual produces an

accelerated vaccinal reaction. The normal individual responds by vaccinia.

Vaccination will be demonstrated; all students desiring it will be vaccinated. The various types of reaction will be shown.

The vaccinal reaction on the skin and in the testicle of the rabbit will be demonstrated. The gross pathology of the vaccinated testicle will be shown.

Microscopic demonstration of vaccine inclusion bodies in the rabbit's cornea..

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BACTERIOLOGIC GLASSWARE

Bacteriologic glassware should be made of strong, hard glass, properly annealed, to withstand the rough handling incident to washing and sterilizing. The glass should be clear, and should be of a quality that does not scale or roughen on repeated sterilization; it should possess a minimum of soluble alkali and should be free from color. Test tubes should be thick-walled, and without lip, to permit close packing in wire baskets. Before use all glassware should be perfectly clean, and free from organic matter, acid and alkali.

New Glassware:

New glassware should be washed with sand-soap and water, and then immersed in 1-2% HCl for a short time and finally rinsed in cold water and dried by draining.

Used Glassware:

Glassware containing cultures should be sterilized in the autoclave. The melted media is then poured out and the glassware washed with soap powder and water while still hot. Glassware containing whole milk, butter or other fatty substances or paraffin should be washed separately, and boiled in soap and water. The final rinsing of all glassware should be with cold water.

Vessels which have been used in the preparation of media must be cleaned immediately after using.

All glassware and in particular pipettes which are in constant use should be cleaned occasionally in the dichromate cleaning solution to remove the film of absorbed material which is gradually deposited and can be removed with difficulty by mechanical means. This solution is made up as follows:

Potassium or sodium dichromate	60 parts
Water	300 parts
Conc. H_2SO_4 (added slowly)	460 or more parts

It should be kept in museum jars or similar glass vessels, with close fitting covers, and should be kept covered to exclude atmospheric moisture. Glassware should remain in this solution for several hours, and then be washed thoroughly in clear water.

Infected pipettes are placed in a tall jar containing lysol (5%) for several hours, and then washed as above. The dichromate cleaning solution is better than boiling in soap and water for pipettes.

Microscopic Slides:

The slides in general use measure 3 inches by 1 inch (76 x 26 mm.) Slides that are to be used repeatedly should be of good white crown glass with ground edges. All slides should be thick enough

to avoid breakage. New slides should always be used for some purposes (e.g. in the examination for tubercle bacilli). New slides should be allowed to remain for some hours in alcohol containing 5% HCl, rinsed in clear water, dried, and polished with old linen. Used slides should be treated with xylol to soften the dried cedar-oil or balsam, then boiled in a solution of soap or washing-soda. If the slide is perfectly clean, a drop of water may be spread over its surface in a thin even film; if it is greasy, a drop of water collects into small droplets and a film cannot be spread.

Cover Slips:

The most useful sizes are the 18 mm. squares or circles for ordinary preparations. Larger rectangular slips are better for covering blood-films and serial sections. Cover slips, which have been used, or are greasy, should be treated as used microscopic slides by boiling in soap or soda solution and then cleaned by dropping into alcohol in an evaporating dish. Concentrated HNO_3 is then added. This should be done under a hood. After a few minutes the acid reacts with the alcohol and fumes of NO_2 are given off. Warming may be necessary to start the process. After thorough rinsing, the slips may be dried and polished, or kept in alcohol and dried as needed. Cover-slips should be handled only by the edges or with forceps. They may be kept ready for use in glass jars intended for the purpose, or in a clean tumbler covered with a Petri plate.

Sterilization of Glassware:

Sterilization by hot air is employed for dry glassware. It is also used for metals subject to rust. It cannot be used for culture media, for rubber or other organic materials.

The dry-air sterilizer should be full each time it is run, for the sake of economy. It is heated by gas. The temperature should be raised gradually to 160°C ., timed for one hour at 160° to 180° , and the gas turned off. It may be run at 180° for 30 minutes, but this temperature makes paper and cotton fibers very brittle. Never open the door until the chamber has cooled below 80°C for fear of breakage due to draughts.

All glassware should be dry before fitting plugs or placing in the sterilizing oven.

Flasks and culture tubes should be plugged with non-absorbent cotton. Two types of plugs are used, rolled and pushed. Rolled plugs are best, but take longer to make. Pushed plugs are adequate for ordinary purposes. Both types will be demonstrated.

A push plug for a standard tube, $150 \times 18 \text{ mm}$. should extend 25 mm. inside the tube and 20 mm. outside. Large plugs for flasks

preserve the same proportions, and should always be of the rolled type. They are most satisfactory if wrapped in gauze or cheesecloth to prevent fraying.

Durham tube vials are placed open end downward in plugged culture tubes. Petri plates are fitted together and stacked cover side uppermost in the metal containers or wrapped in paper in groups of 2 or more plates.

Pipettes are plugged loosely at the mouth end only and placed tip inward in the metal containers. The protruding threads of cotton should be singed off.

Capillary pipettes for qualitative purposes are prepared as follows: A length of glass tubing, 8 mm. diam. x 25 cm. long is plugged with cotton at each end. After sterilization, the tube is softened at the middle in the Bunsen flame and the two ends drawn apart slowly. The capillary portion should be about 40 cm. long. It is softened in the flame, and the two halves separated and sealed.

Steam Sterilization at 100° C.:

This method is extensively employed in bacteriology for the sterilization of culture-media, especially gelatin and those containing sugars, and for articles of rubber. Material to be sterilized is exposed at 100° C for 20 minutes on each of three successive days. The accepted principle of this intermittent or "fractional" method of sterilization is that one exposure is sufficient to kill all vegetative forms of bacteria; between the heatings the spores may pass into vegetative forms, which are destroyed during the subsequent heatings.

The door should always be opened when time is up and the material being sterilized removed at once to avoid wetting the stoppers with water of condensation.

CULTURE MEDIA

Culture media provide the artificial chemical and physical environment necessary for bacterial growth. They supply water, inorganic salts, and carbon, nitrogen and other elements in available form. Different species of bacteria vary greatly in their requirements.

The basis for the media ordinarily employed in the study of the common bacteria is nutrient broth or bouillon. Agar or gelatin is added merely to solidify the medium. To an aqueous extract of meat or beef extract, proteoses and other necessary nitrogenous compounds are added in the form of commercial peptone. The salt content and buffer value of the medium may be increased by the addition of sodium chloride and sodium phosphates. Media may be enriched by the addition of sugars, serum, blood or other substances.

Hydrogen Ion Concentration:

No requirement is more important than the proper hydrogen ion concentration. For the precise determination of the cH of a solution, the electrometric method is employed. Sufficiently accurate results in the measurement of bacteriological culture media may be obtained by colorimetric methods. A comprehensive discussion of the methods may be found in:

CLARK, W. M.: *The Determination of Hydrogen Ions*. 3rd ed., Baltimore, Williams & Wilkins, 1928.

CLARK, W. M., & LUBS, H. A.: *The Colorimetric Determination of Hydrogen Ion Concentration and Its Applications in Bacteriology*. *Jour. Bacteriology* 1917, 2, 1, 109, 191.

A summary will be found in most of the text books.

In applying the colorimetric method, an indicator is selected which has its maximum color change in the neighborhood of the cH to be determined. As standards for comparison one may use a series of solutions of graded pH , with an indicator added, in test tubes; or a printed color chart made to correspond to such tubes may be used, with perhaps more convenience but less accuracy.

For comparison of colored or turbid solutions a special device known as the comparator is useful. This is arranged so that through two apertures in a block of wood one may view light transmitted through the standard tube plus a tube of medium without indicator, and beside, the light through a tube of distilled water plus media with indicator. In this way the alteration of the standard

color by the turbidity and color of the medium is the same in both fields observed.

In well buffered solutions such as culture media, dilution with distilled water does not appreciably alter the pH and is of advantage in reducing the turbidity, lessening the error from the color solution itself and delaying the solidification of agar solutions. In testing pure solutions of strong acids or alkalies, dilution introduces a large error.

To determine the pH of an unknown solution a drop or two of the following indicators may be added to separate small portions of the solution in test tubes.

	<i>Amount of Strength Indicator of added per Solution 10 c.c. of Solution</i>			
	<i>pH Range</i>	<i>Color Range</i>	<i>Used</i>	<i>Solution</i>
Thymol blue (acid range)	1.2-2.8	Red-Yellow	.04%	1.0c.c.
Bromo-Phenol blue	3.0-4.6	Yellow-Blue	.04%	0.5c.c.
Methyl red	4.4-6.0	Red-Yellow	.02%	0.3c.c.
Brom-cresol purple	5.4-7.0	Yellow-Purple	.04%	0.5c.c.
Brom-thymol blue	6.0-7.6	Yellow-Blue	.04%	0.5c.c.
Phenol red	6.6-8.2	Yellow-Red	0.2%	0.5c.c.
Cresol red	7.2-8.8	Yellow-Red	.02%	0.5c.c.
Thymol blue (alkaline range) ..	8.2-9.8	Yellow-Blue	.04%	0.5c.c.

Indicator solutions are prepared by treating the powder with normal alkali before dissolving. For details see Clark, loc-cit.

If the solution is acid to one indicator and alkaline to another, its pH lies between the ranges of these two indicators. An indicator is then selected covering this range and the standard amount indicated in the table is added to 10 c.c. of the solution to be tested. This tube is then compared with a series of tubes of graded hydrogen ion concentration containing the same indicator or with the color chart. The pH of the unknown solution is that of the tube which it most closely matches.

The range of reaction of culture media ordinarily used in the study of pathogenic bacteria is satisfactorily covered by phenol red as an indicator. Take a clear tube containing 8 c.c. of distilled water and add 2 c.c. of the medium to be tested and 0.50 c.c. of phenol red solution (.02% aqueous). To this add very cautiously N/20 NaOH solution from a burette until the color matches that of the standard tube of the reaction desired (for class work pH7.6). Multiply the burette reading by 25 and add this amount of N/1 NaOH to each liter of medium to be corrected.

Mix well and check the correction by determining the pH of the corrected medium.

Titration of Total Acidity:

The determination of total acidity, by titration to an arbitrary end-point (color change of phenolphthalein) was formerly the only means employed for the adjustment of the cH . This method gives a measure of the reserve acidity, and after adjustment to the proper cH , of the reserve alkalinity of a culture medium, and as such is still occasionally of value in determining the buffer value of a medium. It is used according to the directions for the preparation of "standard" media as follows: To 45 c.c. distilled water in a white enameled dish or beaker, add 1 c.c. phenolphthalein solution (0.5 per cent in 50 per cent ethyl alcohol). Boil 1 minute. Test for neutrality by adding 1 drop $\text{N}/20$ NaOH , which should give a distinct purple color in the originally clear fluid. Add 5 c.c. of the media to be tested. Again boil 1 minute. Read the burette. Run into the hot mixture enough $\text{N}/20$ NaOH to produce a faint but distinct and permanent pink color.

Standard methods of water analysis prescribe a color produced by a combination of 25 per cent red (wave length approximately 658) with 75 per cent white on the color top for comparison. Read from the burette the amount of $\text{N}/20$ NaOH used to neutralize the total quantity. Three or more trials should be made until the student can check his previous results within 0.1 c.c. burette reading. Record each result obtaining original data and select those which agree for computation. The burette reading gives the percentage normality directly, since as much $\text{N}/1$ NaOH will be required to neutralize 100 c.c. of media as $\text{N}/20$ to neutralize one-twentieth of 100 c.c. or 5 c.c. The burette reading therefore shows the number of cubic centimeters of $\text{N}/1$ NaOH required to reduce the reaction of 100 c.c. of medium to neutrality to phenolphthalein; but while phenolphthalein is most suitable for the titration of organic acids, its turning point is slightly alkaline to the true neutral point, therefore the reaction of standard media is adjusted, not to neutrality to phenolphthalein, but to $+1$ or $\text{N}/100$ acid. The number of cubic centimeters of $\text{N}/1$ NaOH to be added to every 100 c.c. of media is therefore not that indicated by the burette reading, but that number minus 1. Check the result by repeated titration.

LIQUID MEDIA

1. *Milk.*

Secure freshly separated milk. Test for freedom from excessive acid by heating a tubeful in a bath of boiling water for a few minutes; if it coagulates on heating, it is necessary to add barely sufficient sodium carbonate as determined by titration to prevent coagulation. (If too much alkali is added, subsequent coagulation by bacterial growth is prevented and the usefulness of the medium destroyed; the practice is therefore not generally recommended). When it is shown that the milk does not coagulate on heating it may be tubed and sterilized in the Arnold sterilizer on three successive days for 30 minutes each day. Sterilization in the autoclave renders milk unfit for culture media.

If indication of acid production by bacteria is desired other than that shown by coagulation, sufficient concentrated blue litmus solution, Andrade's indicator, or bromcresol purple may be added previous to sterilization.

2. *Nitrate Solution.*

(1) Mix the following:

0.1 per cent peptone.

0.02 per cent nitrite-free potassium (or sodium) nitrate.

(2) Sterilize in the autoclave.

3. *Dunham's Peptone Solution.*

Peptone (must contain tryptophane for indol test) 10 gms.

NaCl C. P. 5 gms.

Distilled water 1000 c.c.

Dissolve by boiling, filter through paper, and sterilize 30 minutes at 20 pounds pressure in the autoclave.

4. *Meat Extract Bouillon.*

(1) Mix 3 gms. Liebig's Beef Extract.

10 gms. peptone.

5 gms. NaCl.

1000 c.c. distilled water.

(2) Boil over free flame to dissolve.

(3) Restore water lost by evaporation with distilled water to 1000 c.c.

(4) Titrate and if necessary readjust to ± 1 (N/100 acid) to phenolphthalein.

(5) Sterilize in the autoclave under 15 pounds pressure for 30 minutes.

(6) Filter through paper; distribute as required.

(7) Sterilize as in step 5.

5a. *Meat Infusion Bouillon.*

- (1) Stir 1 lb. of very fresh lean chopped beef heart into 1 liter of water. (Distilled preferred.)
- (2) Boil vigorously for 8-10 minutes.
- (3) Strain through cheese-cloth and restore to its original volume with distilled water.
- (4) Add (Bacto) Peptone —1%
NaCl —0.5% and stir until fully dissolved
- (5) Titrate and adjust to pH 7.8-8.0.
- (6) Heat in autoclave at 15 pounds pressure for 10 minutes.
- (7) Filter through one paper until clear.
- (8) Sterilize in autoclave at 15 pounds pressure for 15-20 minutes.

b. *Carbohydrate Broth.*

The above prepared meat infusion after the addition of sugars or other carbohydrates may be used for differential and diagnostic purposes.

- (1) Inoculate a sterile flask of broth with culture of *B. coli* communis and incubate for 24 hours. The bacteria will ferment any monosaccharids which may be present in the broth—thus rendering it sugar free and acid.
- (2) Autoclave for half-hour to kill *B. coli*.
- (3) Add a small amount of technical Kieselguhr to clog the filter for clearing the medium.
- (4) Filter through paper.
- (5) Add 1% of the carbohydrate desired.
- (6) Titrate and adjust to pH 7.8.
- (7) Tube and sterilize in the Arnold for one-half hour on three successive days. The autoclave should not be used in this case because the high temperature will tend to split up the more complex sugars.

c. *Indicators.*

To facilitate the determination of acidity in culture media due to bacterial growth indicators are added, either before sterilization or after growth has occurred.

Litmus Solution is prepared by adding 5% of Merck's purified, or Kahlbaum's litmus to distilled water. To dissolve, the solution must be heated in the Arnold for 2-3 hours, with occasional shaking. It is then filtered through paper and sterilized. It should be kept sterile as molds will grow in it otherwise. It is added to culture medium to a concentration of 3% to 5%.

Andrade Indicator is made up in the following manner:

Acid fuchsin0.5%

Distilled water	100 c.c.
N/1 NaOH	16 c.c.

Filter through paper—add to the medium to a concentration of 1%.

Upon the addition of Andrade, the medium takes on a red color. This color should fade within one to two hours. If it remains red or slightly reddish, the medium should be readjusted to a higher alkalinity.

Acid production turns Andrade Indicator red.

Phenolphthalein Indicator.

The medium should be alkaline to this indicator. Sufficient 1-2% alcoholic solution must be added to produce a faint pink color. This color is discharged when acid is produced.

Other Indicators such as Phenol Red, Brom-cresol-purple and Cresol Red are used. See p. 158. These above indicators may be reduced by bacterial action, so a very small amount should be added after growth has occurred.

6. *Buffered Broth.*

The addition of alkaline phosphates or other salts to liquid medium renders it especially favorable for many delicately growing organisms. 0.5% Na_2HPO_4 or MgCO_3 may be added to meat infusion broth. This obviates the necessity of titration, and if left in excess automatically prevents the accumulation of a high acidity. This medium is eminently suitable for cultivating such organisms as streptococcus, pneumococcus and obligative anaerobes. Sugars should be added aseptically as excessive heating in the presence of a slightly alkaline reaction caramelizes the carbohydrate, thus giving the medium a dark color, and to a degree inhibiting growth.

7. *Gelatin.*

Meat extract bouillon is used as a base for this medium.

- (1) Add 18-22% gelatin.
- (2) Heat in the Arnold occasionally stirring until fully dissolved.
- (3) Titrate and readjust to pH 7.8-8.0.
- (4) Cool to 50°C.
- (5) Add well beaten white of an egg (1 egg for each liter of medium).
- (6) Coagulate by heating in the Arnold for 45 minutes.
- (7) Filter through cotton which has been placed in a steaming hot funnel.
- (8) Tube 12 c.c. each.

- (9) Sterilize in Arnold sterilizer 30 minutes on three successive days.

8. *Meat Infusion Agar.*

This medium is prepared with meat infusion bouillon serving as a base.

- (1) Add to the already prepared broth 2% of agar-agar.
- (2) Heat in autoclave for 40 minutes at 15 pounds pressure to dissolve the agar.
- (3) Cool to 80°C. Titrate and adjust to pH 7.8-8.0.
- (4) Cool to 60°C. and then add well beaten white of one egg to every liter of media.
- (5) Autoclave for 40 minutes at 15 pounds pressure to coagulate the egg albumin.
- (6) Filter through a non-absorbent cotton filter; the funnel should be kept hot by a steam jacket or heating coil.
- (7) Sterilize in autoclave.

9. *Blood and Serum Agar.*

Beside the use of carbohydrates, serum, blood, or albuminous fluids may be added to enrich media for producing growth. One part of the enriching substance is usually added to three parts of the medium under aseptic conditions. In adding enriching substances (blood, serum) to melted agar it must be first cooled to 45°C. Then, in case of a slant, the tube must be placed at the desired angle and left to harden. Blood may be added in the form of whole blood, defibrinated, or citrated blood.

10. *Chocolate Agar*, especially used for influenza bacillus, is made by adding blood to melted agar already cooled to 50°C. and then reheated to 85°C. for 1-2 minutes. It is then allowed to harden.

11. *Endo Medium.*

For the isolation of typhoid and allied bacilli. The basis is ordinary beef extract agar, slightly alkaline to litmus or about pH 8.0. This should be sterilized in bottles in 250 c.c. amounts. When needed, 1% lactose is added and the agar is autoclaved for 20-30 minutes. This serves the double purpose of melting the agar and sterilizing the sugar. Decolorize with 1%-1.5% of a freshly prepared 10% watery solution of sodium sulphite. The color of the agar while fluid should be pinkish and almost colorless when congealed. Plates are poured and allowed to harden. The medium must be mixed each time it is needed and the plates used fresh. Kligler and Defandorfer recommend a reaction of pH 7.6 to 7.8 for the basic agar, the substitution of the bisulphite salt and the addition of 0.5 c.c. of a fuchsin-sulphite mixture (10 c.c. of 10%

NaHSO₃ with 1 c.c. of saturated alcoholic fuchsin), for the *B. dysenteriae* group. Plates should not be poured too hot as the consequent water of condensation will interfere with the isolation of individual colonies.

10. *Eosin Methylene Blue Agar.*

- (1) Prepare meat extract agar adjusted to pH 8.0.
- (2) Add 0.5% each of lactose and saccharose.
- (3) Heat in the Arnold for 10 minutes.
- (4) To 1 liter of agar add 20 c.c. of 2% yellow eosin and 20 c.c. of 2% methylene blue.
- (5) Tube 12 c.c. to a tube and sterilize in autoclave for 20 minutes at 15 pounds pressure.
- (6) When needed melt and pour into sterile Petri dish.

11. *Eosin Brilliant Green Media.*

For use in isolating typhoid organisms from urine.

- (1) To 50 c.c. of already prepared melted meat extract agar add 0.5 grams lactose, 1 c.c. of 3% eosin solution and 1 c.c. of brilliant green solution.
- (2) Tube 15 c.c. to a tube and sterilize in autoclave for 20 minutes, at 15 pounds pressure.
- (3) When needed melt and pour into sterile Petri dish.

12. *Russell's Sugar Media.*

- (1) Prepare one liter of meat extract agar adjusted to pH 7.8.
- (2) Add 1% lactose.
0.1% glucose.
- (3) Add either sufficient litmus to give it the proper color or 1% Andrade Indicator.
- (4) Tube and slant, leaving a generous "Butt" at bottom of the tube for stab inoculation.

It has been found of great help in isolating para-typhoid bacilli to add 1% saccharose. The fermentation of this sugar distinguishes para-typhoid-like bacilli which are frequently found in stools.

13. *Hiss Serum Water Medium.*

- (1) To fresh beef blood serum add three volumes of distilled water.
- (2) Heat in Arnold for 15 minutes.
- (3) Add enough concentrated litmus solution to obtain a deep blue color.
- (4) While hot, add 1% of the carbohydrate desired.
- (5) Test reaction by addition of 1 c.c. N/20 HCl to 5 c.c. of media. A distinct pink should result. If necessary,

adjust with a few drops of N/1 NaOH or HCl, as the case may be.

- (6) Sterilize in Arnold for 25 minutes on three successive days.

14. *Dorset Egg Medium.*

The eggs are thoroughly cleaned with water of any adherent dirt, and then washed with 5% carbolic solution and allowed to partially dry. The ends are then gently dried in the flame and pierced with sharp forceps, which have been flamed. The hole at one end should be about $\frac{3}{8}$ inch in diameter and the membrane broken; at the other end, which is to be blown into, the hole should be smaller and the membrane left unbroken, if possible. The eggs are then blown into a sterile Erlenmeyer flask, the blowing being done from the cheeks. To the egg is then added water (10 per cent by volume of the weight of the eggs). This is mixed by twirling the flask or by gently stirring with a glass rod. Bubbling must be avoided. The mixture is then filtered through cheesecloth by gravity and tubed. The tubes are then slanted and coagulated by heating to 70°C. for two or two-and-a-quarter hours on two successive days. No further sterilization is employed. The medium is incubated to test its sterility.

15. *Petroff's Egg Medium.*

Five hundred grams of beef or veal are infused in 500 c.c. of a 15 per cent solution of glycerin in water, in a cool place. After twenty-four hours the meat is squeezed in a sterile press and the infusion collected in a sterile beaker. The shells of eggs are sterilized by immersion for ten minutes in 70 per cent alcohol. They are broken into a sterile beaker, well mixed and filtered through sterile gauze. One part of meat juice is added to two parts of egg by volume. One per cent alcoholic solution of gentian violet is added to make a final proportion of 1:10,000. The three ingredients are well mixed. The medium is tubed and inspissated as with the Dorset medium.

16. *Lead Acetate Agar.*

- (1) To meat extract agar (1 per cent), adjusted to pH. 7.0 to 7.2 add 1 per cent glucose.
- (2) Tube 5 c.c. to the tube for stabs and sterilize in the autoclave at 15 pounds for 15 minutes.
- (3) While still hot add aseptically to each tube $\frac{1}{2}$ c.c. of a 5 per cent sterile solution of lead acetate and roll.
- (4) This will give a concentration of .05 per cent lead acetate to the resulting media.

17. *Sabourand's Maltose Agar Medium* for the cultivation of fungi.

This medium to be satisfactory must be prepared from crude maltose and to be accurate should be made from ingredients which are obtained at E. Cogit et Cie., Blvd. St. Michel, Paris, France.

Maltose Brute de chanut	40 grams
Peptone de Chassaing	10 grams
Agar	15 grams
Distilled water	15 c.c

The reaction is defined only by the reaction of the special ingredients. It is about pH 6.5.

19. *Casein Digest Medium.*

Medium for the Cultivation of Lactobacillus Group.

- (1) Boil solution containing

H ₂ O	1 liter
Peptone	15 grams
Beef extract	3 grams
- (2) Filter and cool to 60°C.
- (3) Add

Casein	15 grams
Trypsin	3 grams
- (4) Titrate and adjust to pH 7.4.
- (5) Add 5% chloroform to prevent growth.
- (6) Incubate at 37° for 48 hours.
- (7) Boil for several minutes to evaporate the chloroform.
- (8) Add 15 grams of agar and heat to dissolve.
- (9) Titrate and adjust to pH. 7.0.
- (10) Add the whites of eggs; autoclave to coagulate.
- (11) Filter.
- (12) Add 15 grams of lactose.
- (13) 50 c.c. of 2% Sodium Oleate Solution.
- (14) Sterilize.

20. *Sea Water Medium.*

- (1) Obtain sea water.
- (2) Autoclave and filter.
- (3) Add.

Peptone	1%
NaCl	0.5%
Beef extract	0.3%
Agar	2% (if desired)
- (4) Autoclave.
- (5) Titrate and adjust to pH 8.0.
- (6) Filter through same filter until clear.
- (7) Tube and sterilize in autoclave for 25 minutes at 15 pounds pressure.

COMPARATIVE TABLES OF WEIGHTS AND MEASURES

1 inch	=2.54 centimeters.
1 foot	=0.3048 meters.
1 yard	=0.9144 meters.
1 mile	=1.61 kilometers.
1 micromillimeter (micron)	=0.000001 meter=0.00003237 inch.
1 millimeter	=0.001 meter=0.3937 inch.
1 centimeter	=0.01 meter=0.3937 inch.
1 decimeter	=0.1 meter=3.937 inch.
1 meter	=1 meter=39.37 inches=3.28 feet.
1 kilometer	=1000 meters=3281 feet.
1 grain	=0.0648 grams.
1 ounce, avoirdupois	=28.35 grams.
1 ounce, troy	=31.10 grams.
1 pound, avoirdupois	=453.6 grams.
1 pound, troy	=373.2 grams.
1 gram	=15.43 grains.
1 kilogram	=2.205 pounds, avoirdupois.
1 kilogram	=2.679 pounds, troy.
1 gallon, U. S. Liquid	=3785 cubic centimeters.
1 quart, U. S. Liquid	=946 cubic centimeters.
1 pint, U. S. Liquid	=473 cubic centimeters.
1 ounce, U. S. Liquid	=29.57 cubic centimeters.
1000 cubic centimeters	=1.057 U. S. Liquid quarts.

CENTIGRADE AND FAHRENHEIT THERMOMETER SCALES

Centigrade Fahrenheit

—18°	—0.4°	
0°	32°	Freezing
37°	98.6°	Blood heat
56°	132.8°	Temperature of inactivation of sera
60°	140°	Thermal death point of most non-sporulating bacteria
100°	212°	Water boils at sea level, 760 mm. Hg.
107.8°	226°	5 pounds steam pressure
115.5°	240°	10 pounds steam pressure
121.1°	250°	15 pounds steam pressure
126.7°	260°	20 pounds steam pressure

Medical Bacteriology

STAINS AND REAGENTS

The primary step in the preparation of many stains is the making of saturated solutions, which for the following stains contain the designated number of grams of solid stain per 100 cubic centimeters:

	Aqueous	Alcoholic
Methylene blue ¹	6.68	0.66
Gentian violet ¹	1.75	4.42
Basic fuchsin ¹	0.66	2.94

In practice it is customary to add about 20 per cent in excess of these figures, shaking thoroughly and allowing to stand; there should be a slight deposit of dye in the bottom of the bottle, indicating really a supersaturated solution. The supernatant fluid must be filtered for use and any insoluble residue may be restored to the bottle.

1. *Loeffler's Methylene Blue.*²

Saturated alcoholic solution methylene blue	30 c.c.
Aqueous KOH 1-10,000	100 c.c.

2. *Sterling's Modification of Gram's Method.*³

Anilin oil	2 c.c.
Alcohol, 95 per cent.....	10 c.c.
Distilled water	88 c.c.
Gentian violet	5 gms.

Shake anilin oil and alcohol and add 88 c.c. distilled water. Grind gentian violet in a mortar and add anilin solution slowly while grinding. Filter. This solution keeps and stains in one-half to 1 minute.

3. *Gram's Iodine Solution.*

Iodine crystals	1 gm.
Potassium iodide	2 gms.
Distilled water q. s.	300 c.c.

Grind in mortar with 5 c.c. distilled water.

¹ Stitt, Practical Bacteriology, Blood Work, Parasitology. New York, Blakiston.

² Loeffler, Mit. a. d. k. Gesundh., Bd. 2 (1884), S. 421.

³ Gram, Fortschr. d. Med., Bd. 2 (1884), S. 185; U. S. Army Medical War Manual No. 6, p. 27.

4. *Bismarck Brown Counterstain.*

Bismarck brown	10	gms.
Distilled water	1000	c.c.

Boil to dissolve, cool and filter.

5. *Safranin, Counterstain.*

Safranin	5	gms.
Distilled water	1000	c.c.

Boil to dissolve, cool and filter.

6. *Ziehl-Nielson Carbol Fuchsin.*⁴

Basic fuchsin	10	gms.
Ethyl alcohol, 95 per cent	100	c.c.
Phenol, 5 per cent aqueous	1000	c.c.

Dissolve and filter.

7. *Acid Alcohol for Testing Acid-fast Organisms.*

Ethyl alcohol, 95 per cent	800	c.c.
HCl C. P. Conc.	30	c.c.

8. *Neisser Stain for Diphtheria Bacilli.*

(1) Methylene blue	1	gram
Alcohol, 96%	20	c.c.
Glacial acetic acid	50	c.c.
Water	950	c.c.

(2) Bismarck brown 0.2% solution.

9. *Toluidin Blue Stain for Diphtheria.*

Toluidin blue	0.25	gms.
Acetic acid	2.0	c.c.
Absolute alcohol	5.0	c.c.
Distilled water	100.0	c.c.

10. *Giemsa's Stain (old).* Mallory and Wright, Pathological Technique, p. 427.

Azur II eosin	0.3	gm.
Azur II	0.8	gm.
Glycerine	250.	gms.
Methyl alcohol	250.	gms.

11. Nitrite Reagents

1. *Sulphanilic Acid.*

Dissolve 0.5 gm. in 150 c.c. of acetic acid of Sp. Gr. 1.04. (Acetic acid of 1.04 prepared by diluting 400 c.c. of conc. of sp. gr. 1.75 with 700 c.c. of water.)

II. *A-Naphthylamin.*

Dissolve 0.1 gr. in 20 c.c. of water, boil, filter (if necessary) and to clear filtrate add 180 c.c. of acetic acid, Sp. Gr. 1.04.

12. Ehrlich's Indol. Reagent

(A) Paradimethylamidobenzaldehyde	4 parts
Absolute alcohol	380 parts
Concentrated HCl	80 parts

13. Andrade's Indicator.

Acid fuchsin	0.5 gms.
Distilled water	100. c.c.
N/1 NaOH	16. c.c.

Add 1 c.c. per 100 c.c. of media.

14. *Liquor Creosolis Compositus.*

Linseed oil	300 gms.
Cresylic acid	500 gms.
NaOH	54 gms.
Alcohol	30 c.c.
Water to make	1000 c.c.

Heat the oil in a vessel over water bath at 70°C. Dissolve NaOH in 50 c.c. water, warm to 70°C. and add to the oil. Mix thoroughly, add the alcohol, and continue heating till a small portion tested is found soluble in boiling water without the separation of an oily layer. While yet warm add the cresylic acid and mix thoroughly. Keep at 70°C. until a clear solution is produced. Finally add the remainder of the water to make a total volume of 1000 c.c.

This solution can be used in 1 to 2 per cent as a hand wash, and slightly stronger for general disinfection purposes.

CHESTER'S TERMINOLOGY FOR DESCRIPTION OF COLONIES

1. FORM OF COLONIES. PLATE CULTURE

Puntiform: dimensions too slight for defining form by naked eye, minute, raised, semispherical.

Round: of a more or less circular outline.

Elliptical: shape of an ellipse.

Fusiform: spindle-shaped, tapering at each end.

Cochleate: spiral or twisted like a snail shell.

Amoeboid: very irregular, streaming.

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

Filamentous: an irregular mass of loosely woven filaments.

Floccose: of a densely wooly 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.

2. 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.

Punctuate: 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.

3. 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.

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 transversed by veinlike markings as in marble.

Rivulose: marked by lines, like the rivers of maps.

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 *B. anthracis*.

5. EDGES OF COLONIES

Entire: without tothing or division.

Undulate: wavy.

Repand: like the border of an open umbrella.

Erose: as if gnawed, irregularly toothed.

Lobate: border broadly rounded with equally broad sinuses.

Lobulate: minutely lobate.

Auriculate: with earlike lobes.

Lacerate: irregularly cleft, as if torn.

Fimbriate: fringed.

Ciliate: hairlike extensions, radiately placed.

Filamentous: as already defined.

Curled: as already defined.

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 to resin colored.

Translucent: fairly transparent.

Porcelaneous: transparent 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: not transmitting light.

Cretaceous: opaque and white, chalky; dull without luster.

Dull: without luster.

Glistening: shining.

Iridescent: rainbow-like colors.

CLASSIFICATION AND IDENTIFICATION OF MICRO-ORGANISMS

ARTIFICIAL KEY TO THE CLASSES OF PROTOZOA

KINGDOM. ANIMALS.

PHYLUM I. Protozoa—one-celled animals.

Lacking permanent structure for locomotion and nourishment, these functions being performed by means of pseudopodia.

Class 1. SARCODINA

Locomotion by flagellaClass 2. MASTIGPHORA

Locomotion by ciliaClass 3. INFUSORIA

Always parasitic in metazoa, without specialized means for ingestion of food, which is obtained by osmosis—reproduction by sporogonyClass 4. SPOROZOA

For details of further classification consult: Kingsley, Hertwig's Manual of Zoology (New York, Holt). Calkins, Protozoology (New York), Besson, Practical Bacteriology, Microbiology and Serum Therapy; trans. H. J. Hutchins (London, Longmans, 1913).

ARTIFICIAL KEY TO THE CLASSES OF NON-CHLOROPHYLLACEOUS THALLOPHYTA

KINGDOM. PLANTS.

With seedsSubkingdom PHANEROGAMIA

Without seedsSubkingdom CRYPTOGRAMIA

Roots, stems and leaves clearly differentiated.

PHYLUM PTERIDOPHYTA (ferns)

Roots, stems and leaves imperfectly differentiated, archegonia present.

PHYLUM BRIOPHYTA (mosses)

Roots, stems and leaves not differentiated.

PHYLUM THALLOPHYTA (algae, fungi, and bacteria)

With chlorophyll (algae).

Without chlorophyll.

Spores reproductive in function (fungi).*

Reproduced sexually by zygospores or oospores,
asexually by conidia, hypae aerial.

Class HEMIASCOMYCETES

Reproduction by spores, borne in basidia in definite number, eight, in asci, which are always surrounded by a perithecium Class ASCOMYCETES

Reproduction by spores, borne in variable number, in asci of which the perithecium is absent or rudimentary.

Class HEMIASCOMYETES

Reproduction by spores, borne in basidia in definite numbers, mostly four, basidia being either one-celled or cruciately septate Class BASIDIOMYCETES

Reproduction by spores borne on promycelium which may be cruciately septate Class HEMIBASIDIOMYCETES

Reproduction by conidia only, borne either in perithecia or on conidiophores. Life histories in some cases incompletely known and therefore sometimes referred to "fungi imperfecti" CLASS DEUTEROMYCETES

Spores when present resistant in function (bacteria). Reproduction by fission only Class SCHIZOMYCETES

*For detailed study and references consult: Massee, Text book of Fungi (Duckworth & Co., 1906). Brumpt, Précis de Parasitologie (Paris, Masson, 1921). Buchanan, Household Bacteriology (New York, Macmillan, 1915). Anderson, Yeast-like Fungi of the Human Intestinal Tract., Jour. Inf. Dis., vol. 21 (1917), p. 341.

CLASS SCHIZOMYCETES (NAEGELI) MIGULA*

Typically unicellular plants, cells usually small and relatively primitive in organization. The cells are of many shapes, spherical, cylindrical, spiral or filamentous; cells often united into groups, families or filaments; occasionally in the latter showing some differentiation among the cells, simulating the organization seen in some of the blue-green, filamentous alga. Multiplication typically by cell fission. Endospores are formed by some species of the Eubacteriales, conidia by some of the filamentous forms. Chlorophyll is produced by none of the bacteria (with the possible exception of a single genus). Many forms produce pigments of other types. The cells may be motile by means of flagella; some of the forms intergrading with the protozoa are flexuous, a few filamentous forms (as Beggiatoa) show oscillatory movement similar to that of certain blue-green algae (as Oscillatoria).

KEY TO THE ORDERS OF THE CLASS SCHIZOMYCETES (BUCHANAN)

1. Simple and undifferentiated forms, the true bacteria.

ORDER I. EUBACTERIALES

2. Specialized or differentiated forms.

- a. Plant like
- b. Mold like.

ORDER II. ACTINOMYCETALES

- bb. Not mold like.
- c. Sheathed.

ORDER III. CHLAMYDOBACTERIALES

- cc. Not sheathed.
- d. Sulphur bacteria.

* Lohnis and Smith, Life Cycles of the Bacteria, Jour. Agric. Res., vol. 6 (1916), p. 675.

* This attempted classification of the Schizomycetes or bacteria is based on the most recent attempt that has been made to deal with this important and difficult subject. Bergey's Manual of Determinative Bacteriology (Williams & Wilkins, Baltimore, 1923) presents an elaborate classification covering all varieties of bacteria both saprophytic and pathogenic. In the following pages we have attempted to schematize this classification for the pathogens. It is thoroughly realized that any such classification is subject to revision and will undoubtedly be modified, but it seems of advantage to the student to give this outline as indicating modern progress in classification and indicating relationships which should at least be understood in their general aspects.

ORDER IV. THIOBACTERIALES

- dd Slime-mold like.

ORDER V. MYXOMYCETALES

- aa. Protozoan like.

ORDER VI. SPIROCHAETALES

ORDER I EUBACTERIALES

Key to families, tribes and genera of the order Eubacteriales.

I. Organisms obligate aerobes, using oxygen for direct oxidation of carbon, hydrogen or nitrogen or compounds of these. Cells

usually rod shaped, occasionally spherical. Motile or non-motile. Branched involution forms are produced. Endospores never formed. Usually water or earth forms.

Family I. Nitrobacteriaceae (Includes 2 Tribes; 9 Genera; and 30 species).

II. Cells in their free form spherical; during division somewhat elliptical. Division in one, two or three planes. If the cells remain in contact after division they are usually flattened in the plane of division, and occur singly, in pairs, tetrad, packets, chains or in irregular masses. Motility rare. Endospores absent. Metabolism, unlike that of nitrobacteraceae (Family I) is here complex, usually involving the utilization of amino acids or carbohydrates.

Family II. Coccaceae.

Tribe I. Neissereae.

Strict parasites, failing to grow or growing very poorly on usual artificial media. Cells normally in pairs, occasionally in tetrads. Gram negative. Colonies have distinct crumbs scattered on surface.

Genus I. Neisseria Trevisan (7 species).

Species.

- (1) *Neisseria gonorrhoeae*
- (2) " *intracellularis*
- (3) " *catarrhalis*
- (4) " *sicca*
- (5) " *perflava*
- (6) " *flava*
- (7) " *subflava*

Tribe II. Streptococcae.

Parasites (thriving only or best on or in the animal body) except genus *leuconostoc*; grow well under anaerobic conditions. Many forms grow with difficulty on serum-free media, none very abundantly. Planes of fission usually parallel, producing pairs, or short or long chains, never packets. Pigment, if any, orange or white.

Genus II Diplococcus (1 species)

Parasites growing poorly or not at all on artificial media.

Cells usually in pairs.

Type Species.

Diplococcus pneumoniae.

Genus III Leuconostoc (3 species).

Genus IV Streptococcus (24 species).

Chiefly parasites. Normally forming short or long chains. Never in packets. Generally Gram-positive. Inulin rarely attacked. Variable activity in blood.

Species.

I Parasitic or hemiparasitic.

A. *Hemolytic group.*

- (1) *Streptococcus pyogenes*
- (2) " *scarlatinae*
- (3) " *mixtos*
- (4) " *equi*
- (5) " *mastitidis*
- (6) " *cuniculi*
- (7) " *felini*
- (8) " *stenos*

B. *Viridians group.*

- (9) *Streptococcus mitior*
- (10) " *fecalis*
- (11) " *equinus*
- (12) " *bovis*
- (13) " *ignavus*

C. *Colonies gray. No hemolysis or green coloration.*

- (14) *Streptococcus anhemolyticus*
- (13) " *saprophyticus*

II. Saprophytic (9 species)

Genus V. Staphylococcus (6 species).

Usually parasitic. Cells as a rule in irregular groups, rarely in packets. Usually Gram-positive. Growth fair to good on artificial media. Gelatin may be liquified. Nitrates may be reduced. (Produce hemolysis on blood agar.) Pigment white, orange or lemon yellow.

Species.

I. Orange pigment.

- (1) *Staphylococcus aureus*

II. Lemon yellow pigment.

- (2) *Staphylococcus citreus*

III. White or colorless growth on solid media.

- (3) *Staphylococcus epidermidis*
- (4) " *albus*
- (5) " *pharyngis*
- (6) " *tetragenus*

Tribe III. Micrococceae. (3 genera; 42 species).

Facultative parasites or saprophytes, as a rule, aerobic. Grow well on artificial media. Planes of fission often at right angles. Cell aggregates in groups, packets or masses. Generally Gram-positive. Many species form red or yellow pigment.

Family III. Spirillaceae.

Cells elongated, more or less spirally curved. Cell division always transverse, never longitudinal. Cell non-flexuous, usually without endospores. As a rule, motile by means of polar flagella, sometimes non-motile. Typically water forms, though some species are intestinal parasites.

Genus I. Vibrio. (12 species).

(a) Cells short, bent rods, rigid, single or united into spirals. Motile. Polar flagella (1 to 3) or flagellum. Aerobic, facultative anaerobic. No endospores. Usually Gram-negative.

Species.

A. Pathogenic for man or laboratory animals (9 species).

Type: *Vibrio comma* Koch.

B. Non-pathogenic (3 species).

Genus II. Spirillum (4 species).

Family IV. Bacteriaceae.

Rod-shaped cells without endospores. Motile or non-motile. Metabolism complex, amino-acids being utilized and generally carbohydrates. Usually Gram-positive.

Tribe I. Chrombactereae.

Produce pigment on solid media. The pigment may be red, yellow, violet, blue or green.

Genus I Serratia (23 species).

Genus II Flavobacterium (46 species).

Genus III Chromobacterium (9 species).

Genus IV Pseudomonas (20 species).

Tribe II. Achromobactereae.

Non-pigment forming rods, occurring in water and soil. Motile or non-motile. Gram-negative. Rods small to medium in size. Produce a brownish growth on potato.

Genus V Achromobacter (51 species).

Tribe III. Cellulomonadeae.

Short rods occurring in soil, having the property of digesting cellulose. Motile or non-motile. Chromogenic or non-chromo-

genic. Growth in ordinary culture media often not vigorous. Gram-negative. One genus.

Genus VI Cellulomonas (31 species).

Tribe IV. Erwiniae.

Plant pathogens. Growth usually whitish, often slimy. Indol generally not produced. Usually ferment carbohydrates with acid or acid and gas. Motile or non-motile. Gram-negative.

Genus VII Erwinia (11 species).

Genus VIII Phytomonas (36 species).

Tribe V. Zopfiae.

Gram-positive rods, growing freely on artificial media. Do not attack carbohydrates. One genus.

Genus IX Zopfius (2 species).

Species.

1. *Zopfius zopfii*
2. *Zopfius zenkeri*

Tribe VI. Bactereae.

Gram-negative rods generally growing well on artificial media. Generally attack carbohydrates, forming acid and often gas composed of CO_2 and H_2 . When motile the flagella are peritrichous.

Genus X. Escherichia (22 species).

Motile or non-motile rods commonly occurring in the intestinal canal of normal animals. Attack numerous carbohydrates forming acid and frequently acid and gas. Do not produce acetyl-methyl-carbinol.

Type Species.

Escherichia coli

Genus XI Aerobacter (6 species).

Genus XII Proteus (6 species).

Highly pleomorphic rods. Filamentous and curved rods are common as involution forms. Gram-negative. Actively motile possessing peritrichous flagella. Produce characteristic amoeboid colonies on moist media and decompose proteins. Ferment dextrose and sucrose but not lactose. Do not produce acetyl-methyl-carbinol.

Type Species.

Proteus vulgaris

Genus XIII Salmonella (17 species).

Motile forms occurring in the intestinal canal of animals in various inflammatory conditions. Attack numerous carbohydrates with

the formation of acid and gas. In general do not form acetyl-methyl-carbinol.

Species.

(1)	Salmonella	schotmulleri
(2)	"	aertrycke
(3)	"	typhi-murium
(4)	"	veboda
(5)	"	columbensis
(6)	"	enteritidis
(7)	"	psittacosis
(8)	"	abortivo-equina
(9)	"	suipestifer
(10)	"	icteroides
(11)	"	paratyphi
(12)	"	pullora
(13)	"	wolinae
(14)	"	watareka
(15)	"	morgani
(16)	"	guimai
(17)	"	foetida

Genus XIV Eberthella (25 species).

Motile or non-motile rods, occurring in the intestinal canal of man, usually in different forms of enteric inflammation. Attack a number of carbohydrates with the formation of acid but no gas. Do not form acetyl-methyl-carbinol.

Species.

A. Motile (10 species).

Type: *Eberthella typhi*

B. Non-motile (15 species).

Type: *Eberthella dysenteriae*

" para-dysenteriae (Flexner)

" para-dysenteriae (Hiss)

" para-dysenteriae (Strong)

Genus XV Alcaligines (9 species).

Motile or non-motile rods, generally occurring in the intestinal canal of normal animals. Do not form acetyl-methyl-carbinol. Do not ferment any of the carbohydrates.

Type Species.

Alcaligines fecalis

Tribe VII. Encapsulateae.

Short rods, somewhat plump with rounded ends, mostly occur-

ring singly. Encapsulated. Non-motile. Gram-negative. Ferment a number of carbohydrates with the formation of acid and gas. Encountered principally in the intestinal tract of man. Aerobic, growing well on ordinary culture media.

Genus XVI Encapsulatus (6 species).

Type Species.

Encapsulatus pneumoniae

Tribe VIII. Lactobacillae.

Rods, often long and slender. Gram-positive. Non-motile. Without endospores. Usually produce acid from carbohydrates, as a rule lactic. When gas is formed it is CO_2 without H_2 . The organisms are usually somewhat thermophilic. As a rule micro-aerophilic. Surface growth on media is poor.

Genus XVII Lactobacillus (26 species).

Tribe IX. Bacteroideae.

Motile or non-motile rods, without endospores. Show good growth on ordinary culture media; without pigment formation. Obligatory anaerobes.

Genus XVIII Bacteroides (17 species).

Tribe X. Pasteurellae.

Gram-negative rods showing bipolar staining. Parasitic forms with slight fermentative powers. Aerobic, facultative. Powers of carbohydrate fermentation slight; no gas production. Gelatin not liquified. Parasitic, frequently pathogenic, producing plague in man and hemorrhagic septicemia in the lower animals.

Genus XIX Pasteurella (7 species).

Species.

(1)	<i>Pasteurella</i>	<i>avicida</i>
(2)	"	<i>muriseptica</i>
(3)	"	<i>cuniculicida</i>
(4)	"	<i>suisepctica</i>
(5)	"	<i>bovisepctica</i>
(6)	"	<i>tularensis</i>
(7)	"	<i>pestis</i>

Tribe XI. Hemophilcae.

Minute rod-shaped cells, sometimes thread forming and pleomorphic. Non-motile. Strict parasites growing best or only in the presence of hemoglobin and in general requiring blood serum, ascitic fluid, or certain growth accessory substances. Gram-negative.

Genus XX Hemophilii (7 species).

Species

- (1) *Hemophilus influenzae*
- (2) " *hemolyticus*
- (3) " *pertussis*
- (4) " *conjunctivitis*
- (5) " *lacunatus*
- (6) " *ducreyii*
- (7) " *canis*

Genus XXI Dialister (10 species).

Family V. Bacillaceae.

Rods producing endospores, usually Gram-positive. Flagella when present, peritrichous. Often decompose protein medium.

Genus I. Bacillus (75 species). —

Aerobic forms. Mostly saprophytes. Generally liquify gelatin. Often occur in long chains and form rhizoid colonies. Form of rod usually not greatly changed at sporulation.

Type Species.

- (1) Motile
 Bacillus subtilis
- (2) Non-motile
 Bacillus anthracis

Genus II. Clostridium (41 species).

Anaerobes or microaerophiles, often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms.

Pathogenic species.

- (1) *Clostridium welchii* Type I
- (2) " " Type II
- (3) " " Type III
- (4) " " Type IV
- (5) " *egens*
- (6) " *fallax*
- (7) " *oedematis-maligni*
- (8) " *chauvei*
- (9) " *oedematiens*
- (10) " *novyi*
- (11) " *Botulinum* Type A
- (12) " " Type B
- (13) " *histolyticum*
- (14) " *tyrosinogenes*

(15)	"	sporogenes
(16)	"	tetani
(17)	"	lucilae

ORDER II. ACTINOMYCETALES.

Cells usually elongated, frequently filamentous and with a decided tendency to the development of branches, in some genera giving rise to the formation of a definite branched mycelium. Cells frequently show swellings, clubbed or irregular shapes. No pseudoplasmodium. No deposits of free sulphur or iron. No bacterio-purpurin. Endopores not produced but conidia are developed in some genera. Usually Gram-positive. Non-motile. Some species parasitic in animals or plants. Some strongly aerobic and oxidative. Complex proteins frequently required. Growth on culture media often slow; some genera showing mold-like colonies. No water forms.

KEY TO THE FAMILIES OF THE ORDER ACTINOMYCETALES.

- A. Filamentous forms, often branched, sometimes forming mycelia. Conidia sometimes present. Some species parasitic.

FAMILY I. ACTINOMYCETACEA.

- B. Parasitic forms. Rod-shaped, rarely filamentous, and with only slight and occasional branching. No conidia.

FAMILY II. MYCOBACTERIACEAE.

Family I. Actinomycetaceae.

Filamentous forms, often branched and sometimes forming mycelia. Conidia sometimes present. Some species are parasitic.

Genus I. Actinobacillus (10 species).

Genus II. Leptotrichia (1 species).

Thick, long, straight or curved filaments, unbranched, frequently clubbed at one end and tapering to the other. Gram-positive when young. Filaments fragment into short, thick rods. Anaerobic or facultative. No aerial hyphae or conidia. Parasites or facultative parasites.

Type Species.

Leptotrichia buccalis.

Genus III. Actinomyces (64 species)

Organisms growing in form of much-branched mycelium, which

may break up into segments which function as conidia. Sometimes parasitic, with clubbed ends or radiating threads conspicuous in lesions in the animal body. Some species are microaerophilic or anaerobic. Non-motile.

Type Species.

Actinomyces bovis.

Genus IV. Erysipelothrix (1 species).

Rod-shaped organisms with a tendency to the formation of long filaments which may show branching. The filaments may also thicken and show characteristic granules. No spores. Non-motile. Gram-positive. Do not produce acid. Microaerophilic. Usually parasitic.

Type Species.

Erysipelothrix rhusiopathae

Family II. Mycobacteriaceae.

Parasitic forms. Rod-shaped, frequently irregular in form but rarely filamentous and with only slight and occasional branching. Often stains unevenly (showing variations in staining reaction within the cell). No conidia formed.

Genus I. Mycobacterium (17 species).

Slender rods which are stained with difficulty, but when once stained are acid-fast. Cells sometimes show swollen, clavate or cuneate forms and occasionally even branched forms. Growth on media slow. Aerobic.

Type Species (Parasitic in man).

Mycobacterium tuberculosis

“*leprae*

Genus II. Corynebacterium (16 species).

Slender, often slightly curved rods with a tendency to club and pointed forms, with branching forms in old cultures. Barred, uneven staining. Not acid-fast. Gram-positive. Non-motile. Aerobic. No endo-spores. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when cells divide.

Type Species.

Corynebacterium diphtheriae

Genus III. Fusiformis (2 species).

Obligate parasites. Anaerobic or microaerophilic. Cells frequently elongate and fusiform, staining somewhat unevenly. Filaments

sometimes formed; non-branching. Non-motile. No spores formed. Growth in laboratory media feeble.

Type Species.

Fusiformis dentium

Genus IV. Pfeifferella (1 species.)

Non-motile rods, slender, Gram-negative. Staining poorly, sometimes forming threads and showing a tendency toward branching. Gelatin may be slowly liquefied. Do not ferment carbohydrates. Growth on potato characteristically honey-like.

Type Species.

Pfeifferella mallei

ORDER III. CHLAMYDOBACTERIALES.

Filamentous bacteria, algae-like typically water forms, frequently sheathed, without true branching although false branching may be present. The sheath is frequently impregnated with iron. Conidia may be developed but never endospores. Sulphur granules or bacteriopurpurin never present. Mature cells or filaments not motile or protozoan like.

Family I. Chlamydobacteriaceae.

Genus I. Leptothrix (4 species).

Four other non-pathogenic Genera; 5 species

ORDER IV. THIOBACTERIALES.

Cells various, typically containing either granules of free sulphur or bacteriopurpurin, or both, usually growing best in the presence of hydrogen sulphide. The cells are plant-like, not protozoan-like, not producing a pseudoplasmodium or a highly developed resting stage. Spores are rarely or never formed. There are listed in Bergey under this Order, 3 families, 2 sub-families, 5 tribes, 30 genera and 62 species.

ORDER V. MYXOBACTERIALES.

Motile, rod-like organisms multiplying by fission, secreting a gelatinous base and forming a pseudoplasmodium-like aggregation before passing into a more or less highly developed cyst-producing, resting stage in which the rods may become encysted in groups without modification, or may be connected into spore masses. (1 family; 3 genera; 21 species.)

ORDER VI. SPIROCHAETALES.

Protozoan-like in many characteristics. Cells usually relatively

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