LABORATORY EXERCISES IN GENERAL ELEMENTARY BACTERIOLOGY

By

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To
Miss Williams

-from M. Spiegel.

PREFACE

The laboratory exercises given in this manual are those which the writers give to their students in General Elementary Bacteriology, a course in which the students spend in the laboratory three periods a week of two and one-half hours each during one semester. Only those exercises are included which are actually performed by the students. An especial effort has been made to include only such methods as give definite and satisfactory results in the hands of the students.

No apology is offered for the selection of material included in these exercises, though it is of course recognized that equally satisfactory selections might be made which would vary greatly in nature and scope from the subject matter here included. It is recognized that many important aspects of bacteriology, which are necessarily covered in the class-work in a course in General Bacteriology, are entirely omitted in these laboratory outlines. The exercises are intended to serve only as a laboratory guide and not in any sense to take the place of the instruction and personal direction which must be given in the laboratory.

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PART 1

GENERAL METHODS

SECTION 1: MORPHOLOGY OF MICROORGANISMS

EXERCISE 1

THE MICROSCOPE

Make a detailed study of the microscope and note carefully its important features and its operation, as demonstrated and explained. Also study the booklet on "The Microscope", published by the Spencer Lens Company. For a more comprehensive study of the microscope and its uses see "The Microscope", by Gage.

EXERCISE 2

MICROSCOPIC EXAMINATION OF INANIMATE OBJECTS

Make microscopic studies and drawings of the following materials:

(1) Particles of fine sand
(2) Crystals of sodium chloride
(3) Cotton fibers
(4) Wool
(5) Fat globules of milk

Examine the sand particles and salt crystals in the dry state, using the low magnification (16 m.m. objective) of the microscope. After observing the salt crystals using the low power, examine them under the 4 m.m. objective.

Using wet preparations, examine the cotton and wool under the 16 m.m. and 4 m.m. objectives.

Dilute some whole milk with water, using about one part of milk to five parts of water. Examine this liquid under the 16 m.m. and 4 m.m. objectives. Also observe the Brownian movement of the globules.

EXERCISE 3

EXAMINATION OF STAINED BACTERIA

Study and make drawings of prepared slides showing different morphological types of bacteria. Examine under the oil immersion objective. Stained slides of the following organisms are furnished:

(1) *Staphylococcus aureus*
(2) *Streptococcus pyogenes*
(3) *Sarcina lutea*
(4) *Bacterium typhosum*
(5) *Bacillus anthracis*
(6) *Vibrio cholerae*
(7) *Spirillum rubrum*
(8) *Actinomyces bovis*

EXERCISE 4

MICROSCOPIC MEASUREMENTS

By use of a stage micrometer, determine the value of the divisions on your ocular micrometer. Having calibrated the ocular micrometer, make measurements of a number of representative bacteria found on your stained slides. More delicate measurements may be obtained with the aid of a filar micrometer ocular.

EXERCISE 5

EXAMINATION OF LIVING BACTERIA

Hanging Drop Preparation

Clean a cover glass and place it in a pair of forceps. Clean a hanging drop slide and spread vaseline around the edge of the depression. Place a small drop of the infusion in the center of the cover glass. Quickly invert the forceps and cover glass so that the drop hangs. Carefully place the cover slip with the hanging drop over the depression. Press down the cover slip on the slide in order to seal and thus prevent evaporation. See that the drop hangs from the cover glass without touching the side or bottom of the cavity.

Exclude most of the light by means of the iris diaphragm and examine first under the 16 m.m. objective to locate the edge of the drop. Having found this, raise the tube and swing the 4 m.m objective into place. While watching the objective from the side, run it down until the tip of the objective almost comes in contact with the cover slip; then slowly and carefully focus upwards, regulating the light in the meantime by means of the iris diaphragm, until the bacteria come into view.

Study carefully. Note especially the difference between true motility and Brownian movement. Make drawings.

Cover Glass Preparation

While not so satisfactory as the hanging drop method, living bacteria may be observed by simply placing a drop of the culture on an ordinary slide and covering it with the cover glass.

EXERCISE 6

MORPHOLOGY OF MOLDS, YEASTS, AND PROTOZOA

Make a wet preparation of the culture of yeast furnished. Make drawings. Examine the mold cultures, furnished on agar plates, under the 16 m.m

objective. Focus on the edge of the colony and study its structure. Place a cover glass over the growth and examine under the 4 m.m. objective. Note the characteristics of the hyphae, the reproductive cells and spores. In addition make a wet preparation from the mold growth and examine under the 4 m.m. objective. Make drawings.

Examine the organic infusions for protozoa. Note the different types observed and identify representatives of the three main groups—amoebae, ciliates, and flagellates. Make drawings.

SECTION 2: SIMPLE STAINING TECHNIQUE

EXERCISE 7

PREPARATION AND FIXING OF BACTERIA FOR STAINING

(1) Using the wire loop, place a small drop of broth culture on a clean slide. If the culture is taken from solid media, place a small drop of water on the slide and thoroughly mix with it a small bit of the culture growth.

(2) Spread the drop on the slide to form a thin film.

(3) Allow the film on the slide to dry in the air or by holding the slide high above the flame.

(4) When the film is dry pass the slide, film side up, three times through the Bunsen flame.

The heat kills the organisms and causes them to adhere to the slide. This process is termed "fixing" the preparation. The slide so prepared is ready for staining.

EXERCISE 8

METHYLENE BLUE STAIN

Make stained preparations of the cultures furnished and also of saliva, using a solution of methylene blue.

Procedure

(1) According to the method outlined in Exercise 7, fix the slide.

(2) Place the slide on the wire staining screen over the waste jar.

(3) Drop on enough of the methylene blue solution to cover well the film.

(4) Allow the stain to act about 30 seconds.

(5) Wash the stained preparation with water from the wash bottle.

(6) Dry the slide between blotting paper.

(7) Examine the stained preparation under the microscope and make a drawing of a good field.

Formula

Methylene blue, U.S.P. medicinal, saturated alcoholic solution..... 30 c.c.
Potassium hydroxide, 0.01% aqueous solution.....100 c.c.

EXERCISE 9

GENTIAN VIOLET STAIN

Using a solution of gentian violet make stained preparations from the same materials as used in Exericse 8. Make drawings.

Procedure

Use the same procedure as in Exercise 8, except step (4). In step (4) allow the stain to act about ten seconds.

Formula

Gentian violet, saturated alcoholic solution.....10 c.c.
Ammonium oxalate, 1.0% aqueous solution.....40 c.c.

EXERCISE 10

CARBOL-FUCHSIN STAIN

Make stained preparations from the same materials as used in Exercises 8 and 9, using a solution of carbol-fuchsin. Make drawings.

Procedure

Use the same method as followed in Exercises 8 and 9, except steps (3) and (4).

In step (3) moisten the film with water, then apply an equal amount of the staining solution.

In step (4) allow the stain to act five to fifteen seconds.

As carbol-fuchsin is even a more powerful stain than gentian violet difficulty may be experienced from overstaining, especially in preparations which contain large amounts of organic matter and foreign debris.

Formula

Basic fuchsin, saturated alcoholic solution..... 3 c.c.
Phenol, 5.0% aqueous solution.....19 c.c.

EXERCISE 11

PREPARATION OF PERMANENT SLIDES

Make stained preparations and mount in Canada balsam on a slide, as demonstrated.

SECTION 3: INDIRECT STAINING METHODS

Exercise 12

INDIA INK PREPARATION

A useful method for examining bacteria under the microscope is to suspend them in India ink. When this method is used, the bacteria remain unstained in a dark background. By the use of India ink it is possible to examine any organism unstained. It has the advantage that the organisms do not shrink or change their form.

Procedure

Place a drop of India ink on a microscopic slide. Mix with this a small amount of the culture to be examined. Spread the preparation to a thin uniform film and allow to dry. Using the oil immersion objective, examine the preparation and make drawings.

Exercise 13

NIGROSINE PREPARATION

As it is difficult to obtain an India ink preparation which is satisfactory nigrosine is recommended as a better substance for indirect or negative staining. A one per cent aqueous solution of nigrosine is used.

Procedure

Place a small drop of the nigrosine solution on a slide, mix with it a small loopful of a broth culture and spread well. Allow to dry before examination under the microscope. Make drawings.

Note

Nigrosine may also be used to demonstrate motility in bacteria. For this purpose, add a very small amount of the nigrosine solution to a hanging drop.

Exercise 14

CONGO RED PREPARATION

Make a slide preparation as in Exercise 13, using a saturated aqueous solution of congo red instead of nigrosine. When the preparation has dried, immerse the slide in a 95 per cent alcoholic solution containing one per cent hydrochloric acid. The bacteria will be unstained on a deep blue background. By use of the oil immersion, study and make drawings.

SECTION 4: THE CULTURE OF BACTERIA

EXERCISE 15

BROTH CULTURES

Treat three tubes of nutrient broth, or bouillon, as follows:

(1) Label one tube "Control" and leave uninoculated. Do not remove the cotton plug.

(2) Remove the cotton plug from one tube and add a small amount of dirt or other foreign material. Replace the cotton plug.

(3) With the wire loop inoculate the third tube of broth from the *pure culture furnished.*

Observe carefully the technique used and the precautions taken in making transfers.

Incubate at 37° C. The inoculating needle should always be heated to redness, and the lower end of the handle passed through the flame several times, *immediately before* and *after* making a transfer. Also, the mouths of the tubes from which cultures are taken and into which they are transferred should be passed through the Bunsen flame *immediately before* and *after* the needle is introduced and removed. After incubation for two days, make observations.

EXERCISE 16

AGAR SLOPE CULTURES

Melt two tubes of nutrient agar in boiling water. When melted, cool to a temperature of 42° to 45° C. Remove from water and allow to harden in a slanting position.

When cold and solid, make a streak culture, as demonstrated, from the pure culture furnished. Keep the other tube sterile as a control. Incubate at 37° C.

In inoculating slope cultures the needle should be drawn gently over the surface of the agar from the butt to the top of the sloped surface. Care should be taken not to scratch the agar surface. The same technique and precautions as explained in Exercise 15 should always be observed in the handling of cultures.

Observe after incubation for two days.

EXERCISE 17

PLATE CULTURES

Melt four tubes of nutrient agar in boiling water. When thoroughly melted, cool to 42° to 45° C. While in a melted condition pour the agar into sterile Petri dishes.

In pouring the agar from the tube into the Petri dish care should be taken to prevent contamination. When the melted agar is removed from the water bath the outside of the tube should be wiped with a cloth to remove the adhering water. When the cotton plug is removed to pour the agar, the mouth of the tube should be flamed to kill microorganisms on the outside lip of the tube. In pouring the agar from the tube to the plate, the cover of the plate should be raised only on one side, and just sufficiently to admit easily the mouth of the tube. Care should be taken not to scrape the tube on the dish or its cover when pouring the agar. When the agar has been poured into the plate, lower the cover into place immediately, pick up the dish and gently tilt from side to side in order to make a uniform layer of agar over the entire bottom. Replace the plate on the table and allow the agar to harden. Care should be taken not to move the plate until the agar is thoroughly solidified and cold.

Treat the agar plates prepared as follows:

(1) Allow one to remain sterile as a control.

(2) Remove the cover and allow one to remain exposed to the air of the laboratory for fifteen minutes before replacing the cover.

(3) Remove the cover from one dish and touch the surface of the agar lightly with the tips of your fingers.

(4) By means of your wire loop, streak some saliva on the surface of the agar of the remaining plate. Note carefully the technique of this operation as demonstrated. Especial care should be taken not to break the surface of the agar when streaking.

After the plates are prepared and thoroughly hardened, they should be inverted before incubation.

Incubate at 37° C. After two days, make observations.

SECTION 5: DIFFERENTIAL STAINING TECHNIQUE

EXERCISE 18

GRAM STAIN

Make Gram stains of the cultures furnished.

Procedure

(1) Fix the preparation.

(2) Stain with gentian violet for about 30 seconds.

(3) Rinse with water.

(4) Cover the film with Gram's iodine and allow it to act for about 30 seconds.

(5) Rinse with water.

(6) Decolorize in 95% alcohol. Thirty to 60 seconds is long enough for a thin smear. Decolorization should be continued until the color ceases to run from the film.

(7) Rinse with water.

(8) Counterstain with safranin or Bismark brown for ten to 30 seconds.

(9) Rinse with water and blot dry.

(10) Examine under the oil immersion objective. Make drawings.

Gram positive organisms stain a purplish black; Gram negative organisms take the color of the counterstain.

Formulae

Gentian Violet Solution

Gentian violet, saturated alcoholic solution ..... 10 c.c.
Ammonium oxalate, 1.0% aqueous solution ..... 40 c.c.

Gram's Iodine Solution

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

Bismark Brown Solution

Saturated alcoholic solution ..... 1 c.c.
Distilled water ..... 10 c.c.

Safranin Solution

Saturated aqueous solution

Exercise 19

ACID-FAST STAIN

Make stained preparations of the cultures furnished and also of tubercular sputum.

Procedure

(1) Fix the slide.
(2) Stain the fixed smear for five minutes by placing the slide in a receptacle containing carbol-fuchsin heated by boiling water.
(3) Rinse with water.
(4) Decolorize for one to three minutes in acid alcohol.
(5) Wash thoroughly with water.
(6) Counterstain with methylene blue for thirty seconds to one minute.
(7) Examine under the oil immersion objective and make drawings.

The tubercle organisms will be stained red. Any non-acid-fast organisms present will be stained blue.

The carbol-fuchsin and methylene blue solutions used for this purpose are the same as those employed in previous exercises for simple stains. The acid alcohol solution contains 3% hydrochloric acid in 95% alcohol.

Exercise 20

SPORE STAIN

Make stained preparations of the culture furnished.

Procedure

Follow the same procedure as outlined in Exercise 19, except in step (4).

In step (4) decolorize in 95% alcohol until most of the color is removed.

When this method is carried out successfully, the spores will have a distinct red color and the rest of the organisms will be blue.

Exercise 21

SPORE STAIN: DORNER'S METHOD

Make stained preparations of the cultures furnished.

Procedure

(1) In five drops of sterile water in a test tube make a very heavy suspension of the organism.
(2) Add an equal quantity of carbol-fuchsin.
(3) Allow to stain in boiling water bath for ten minutes.
(4) From these stained organisms make a nigrosine preparation.
(5) Examine under the oil immersion objective and make drawings.

By this method the spores will have a red color and the rest of the organisms are seen unstained in a dark background.

EXERCISE 22

CAPSULE STAIN

Make stained preparations of the cultures furnished.

Procedure

(1) Fix the slide.

(2) Cover the film with glacial acetic acid for a few seconds.

(3) Shake off the excess of the glacial acetic acid.

(4) Add portions of the aniline-water gentian violet until all the acid is washed out.

(5) Wash in a 2% solution of sodium chloride and mount in the same solution. Do not let water come in contact with the film at any time.

When this method of staining is successful, the bodies of the bacteria show a deep violet color and the capsules a lighter, pinkish purple color.

Formulae

Aniline Water

Aniline.....1 part
Distilled water.....9 parts

Aniline-Water Gentian Violet

Aniline water.....9 parts
Gentian violet, saturated alcoholic solution.....1 part

EXERCISE 23

CAPSULE STAIN: INDIA INK METHOD

Another method for determining the presence of capsules on bacteria may be used.

Procedure

(1) Place a drop of India ink upon a microscopic slide.

(2) Put a small amount of the culture to be examined into the drop of ink and spread it into a thin film.

(3) Fix the slide.

(4) Stain the preparation in the customary way by using the simple carbol-fuchsin or the Gram stain.

(5) Observe and draw.

When bacteria are stained by this method the bodies of the organisms are the color of the stain used and the capsules are unstained in a dark background.

EXERCISE 24

FLAGELLA STAIN: CASARES-GIL'S METHOD

A number of methods have been described for staining flagella. While none of these methods has been entirely satisfactory, the method of Casares-Gil as modified by Mrs. Barber is probably as reliable as any.

(1) Mordant:

Tannic acid	10 grams
Aluminum chloride (hydrated)	18 grams
Zinc chloride	10 grams
Rosanilin hydrochloride	1.5 grams
Alcohol (60%)	40 c.c.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 c.c. of the alcohol first, and then the rest slowly. This alcoholic solution may be kept several years. For use, dilute with two parts of water, filter off precipitate and collect filtrate on the slide allowing it to act for 60 seconds.

(2) Stain: Carbol-fuchsin.

SECTION 6: METHODS OF STERILIZATION

EXERCISE 25

PREPARATION OF MATERIALS FOR STERILIZATION

Prepare Petri dishes, pipettes, empty flasks and test tubes, and test tubes containing broth, for sterilization. Note carefully the details of the methods used in the preparation of glassware for sterilization.

EXERCISE 26

METHODS OF STERILIZATION

For methods of sterilization see "Bacteriology", Buchanan, pages 105-110. Note the various methods of sterilization other than those used in Exercises 27, 28, and 29.

EXERCISE 27

STERILIZATION BY DRY AIR

Sterilize the pipettes and Petri dishes prepared in Exercise 25 in the hot-air sterilizer. Use a temperature of 170° C. Hold for one hour after this temperature is reached.

Is dry or moist heat more efficient as a sterilizing agent? Why? In this connection also read in "Bacteriology" by Buchanan, pages 172-174.

EXERCISE 28

STERILIZATION BY STEAM UNDER PRESSURE

Sterilize some of the tubes of broth, prepared in Exercise 25, in the autoclave. Use a pressure of 15 pounds for 30 minutes. Steam under a pressure of 15 pounds has a temperature of about 121° C. Note carefully the steps and precautions to be taken in the operation of the autoclave.

The time necessary for complete sterilization in the autoclave will vary somewhat according to the nature of the material, and the amount of material in the container.

EXERCISE 29

INTERMITTENT STERILIZATION

Sterilize some tubes of broth in flowing steam (100° C.) by the intermittent or discontinuous method. Heat for 30 minutes on each of three successive days, allowing them to remain at laboratory temperature between heatings. For a more complete description of this process, and the principles upon which it is based, see "Bacteriology" by Buchanan, pages 105 and 106.

SECTION 7: PREPARATION OF CULTURE MEDIA

EXERCISE 30

COLORIMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION

Determine the hydrogen ion concentration of the samples of media furnished, using standard buffer solutions. Also use color charts in the place of the standard buffers.

First, by use of the spot plate determine the correct indicator solution to add to the medium being tested.

Procedure

Place 5 c.c. of the medium in a clean test tube and dilute with 5 c.c. of distilled water. Add 5 drops of the indicator solution and mix thoroughly. Match the color obtained with the proper tube in the standard buffer series.

The accuracy of this method may be increased by the use of a comparator block which compensates for the turbidity of the solution under test. Observe carefully the method of using the comparator block.

COLOR CHANGES OF INDICATORS USED IN DETERMINING pH

Indicators	Full acid color	Full alkaline color	Sensitive range pH values
Thymol blue (T.B.) (acid range)	red	yellow	1.2—2.8
Brom phenol blue (B.P.B.)	yellow	blue	3.0—4.6
Brom cresol green (B.C.G.)	yellow	blue	3.8—5.4
Methyl red (M.R.)	red	yellow	4.4—6.0
Chlor phenol red (C.P.R.)	yellow	red	5.0—6.6
Brom cresol purple (B.C.P.)	yellow	purple	5.4—7.0
Brom thymol blue (B.T.B.)	yellow	blue	6.0—7.6
Phenol red (P.R.)	yellow	red	6.6—8.2
Cresol red (C.R.)	yellow	red	7.2—8.8
Meta cresol purple (M.C.P.)	yellow	red	7.4—9.0
Thymol blue (T.B.)	yellow	blue	8.2—9.8
Phenolphthalein	Colorless	red	8.0—9.6
Cresolphthalein	Colorless	red	8.2—9.8

EXERCISE 31

ADJUSTMENT OF CULTURE MEDIA REACTION

After determining the hydrogen ion concentration, adjust 5 c.c. portions of the media furnished to a reaction of pH 7.0 by the addition of N/20 NaOH (or

N/20 HCl if the original reaction is more alkaline than pH 7.0) using bromthymol blue as indicator. From the amount of N/20 NaOH (or N/20 HCl) necessary to adjust 5 c.c. to pH 7.0, calculate how much N/1 NaOH (or N/1 HCl) would be required to adjust one liter of the same medium to a reaction of pH 7.0.

By the same methods adjust 5 c.c. portions of different media to a reaction of pH 5.2, using methyl red as indicator, and calculate the required amounts of N/1 NaOH or N/1 HCl to adjust one liter amounts of the same media to a reaction of pH 5.2.

If end points of pH 9.0, pH 8.0, pH 7.4, pH 6.2, and pH 4.2 were desired, which indicators would you select?

EXERCISE 32

NUTRIENT BROTH OR BOUILLON

Prepare one liter of nutrient broth using the following formula:

0.3% beef extract
0.5% peptone
Distilled water
Reaction, pH 7.0

Procedure

(1) Mix the above ingredients and steam for about 10 minutes to dissolve the ingredients.

(2) Determine the hydrogen ion concentration and adjust to the desired reaction.

(3) Heat in the autoclave under 15 pounds pressure for 10 minutes in order to precipitate heat coagulable substances.

(4) Filter through paper.

(5) Measure and restore any loss from evaporation by the addition of distilled water.

(6) Distribute in the desired containers, and sterilize in the autoclave at 15 pounds pressure for 30 minutes.

EXERCISE 33

NUTRIENT AGAR

Formula

0.3% beef extract
0.5% peptone
1.5% agar
Distilled water
Reaction, pH 7.0

Procedure

Follow the same procedure as in making bouillon with the following differences: In step (1) the ingredients should be dissolved by heating in the autoclave under 15 pounds pressure for 15 minutes. In step (4) absorbent cotton may be used for filtration instead of paper.

EXERCISE 34

NUTRIENT GELATIN

Formula

0.3% beef extract
0.5% peptone
12.0% gelatin
Distilled water
Reaction, pH 7.0

Procedure

Use the same procedure as in the preparation of nutrient agar except: The heating in steps (1) and (3) should be in flowing steam instead of in the autoclave. Sterilization should be done by the intermittent method at 100°C. Gelatin may be sterilized in the autoclave in which case it should be cooled immediately upon removal.

EXERCISE 35

BEEF INFUSION BROTH OR BOUILLON

For many purposes nutrient media prepared with fresh meat are superior to those made with beef extract. This is especially true for the growth of a number of the pathogenic microorganisms.

Formula

500 grams lean ground beef per liter
1.0% peptone
Distilled water
Reaction, pH 7.0

Procedure

(1) Add 500 grams of ground lean beef to 1000 c.c. of distilled water and mix thoroughly.

(2) Hold in a refrigerator at about 10° C. for 24 hours.

(3) Remove the meat from the bouillon by straining through cheesecloth.

(4) If necessary, remove the excess fat.

(5) Add one per cent of peptone and dissolve.

(6) Adjust the reaction.

(7) Heat in the autoclave for 15 minutes under 15 pounds pressure.

(8) Filter through paper and make the solution up to 1000 c.c. by adding distilled water.

(9) Sterilize in the autoclave for 30 minutes at 15 pounds pressure.

A solid medium can of course be prepared by the addition of agar or gelatin to this broth.

Exercise 36

CARBOHYDRATE MEDIA

The addition of carbohydrates and related compounds (simple sugars, polysaccharids, polyhydric alcohols, and glucosides) to culture media is a common practice in bacteriological work. Usually such media are simply the standard nutrient media described in Exercises 32 to 35 with one per cent of the particular carbohydrate added.

In the preparation of carbohydrate media, especially in the cases of simple sugars, the carbohydrate should be added to the rest of the ingredients just prior to filtration and sterilization in order to obviate unnecessary heating, as there is some danger of slight hydrolysis of the sugar at high temperatures, especially in the presence of the other constituents of the medium. For this reason, in cases in which particular accuracy is desired, solutions of the sugars are sometimes sterilized separately and then added to the medium just before use.

It is also a common practice to use indicators in carbohydrate media to detect the presence of specific organisms which have the power of fermenting the carbohydrate in question; for example, the use of lactose agar with the addition of an indicator, such as litmus or brom cresol purple, for the detection and isolation of lactose fermenting bacteria in water or milk.

SECTION 8: PHYSIOLOGICAL REACTIONS OF BACTERIA

EXERCISE 37

GAS FORMATION

Using the culture of *Bacterium coli* furnished, inoculate:

(1) A Smith fermentation tube containing glucose broth.
(2) A Durham fermentation tube containing glucose broth.
(3) A tube of glucose broth covered with a paraffine jelly seal.
(4) A glucose agar "shake culture".

Melt the tube of glucose agar in boiling water. When melted, cool to a temperature of 42° to 45° C. and inoculate. Thoroughly mix the inoculum with the medium, being careful to introduce as little air as possible. Cause the agar to solidify quickly by placing the tube in cold water.

Incubate at 37° C. At the next period notice the collection of gas.

The gas produced by most gas-forming bacteria consists chiefly of carbon dioxide and hydrogen. Analyze the gas formed in the Smith fermentation tube for carbon dioxide in the following manner.

Measure on the arm of the fermentation tube the amount of gas formed. Fill the bulb with a 2% solution of NaOH and close the mouth with the thumb, taking care not to leave any air between the thumb and the liquid.

Tilt the gas back and forth slowly from the closed arm to the bulb and back to the closed arm five or six times and finally allow the gas to collect again in the closed arm. The NaOH combines with the carbon dioxide and, consequently, on releasing the thumb, the volume of gas will become smaller in proportion to the amount of carbon dioxide absorbed.

Measure the amount of gas again and note the relation of carbon dioxide to the total gas formed.

Test the remaining gas for hydrogen by tilting it back into the bulb, previously covered with the thumb, and then holding the mouth of the tube to a flame and releasing the thumb. A slight explosion takes place from the combination of the hydrogen with the oxygen of the atmosphere.

Give the ratio of hydrogen to carbon dioxide found according to your results. The ratio of these two gases formed varies with different organisms. The results obtained from the quantitative analysis of the gas in a Smith fermentation tube are not accurate since a large amount escapes from the open end of the tube. The proportion of carbon dioxide to hydrogen as revealed by this test is not accurate as the carbon dioxide is soluble and, therefore, a larger proportion of it is lost from the open end of the tube than of the insoluble hydrogen. However, the testing of the gas produced in a Smith fermentation tube is of value for qualitative purposes.

EXERCISE 38

THE FERMENTATION OF CARBOHYDRATES

The fermentation of carbohydrates, higher alcohols, and glucosides, with the production of acids (and gas in the case of some organisms) is a very valuable reaction in the study of bacteria. Since some organisms ferment a great many of these substances, while others ferment only a few, and still others do not ferment any of them, this reaction is frequently of great value in the differentiation and identification of bacteria. Most of the substances which have been used for studying the fermentation reactions of bacteria are given below. Some of these are only rarely used for this purpose, while others are frequently used.

Inoculate glucose, lactose, and sucrose broths, contained in Durham tubes, with cultures of *Streptococcus lactis, Bacterium coli, Bacillus subtilis* and *Proteus vulgaris*. Incubate at 37° C. Observe after two days.

The broth furnished contains brom cresol purple as an indicator of acid formation.

CARBOHYDRATES

Monosaccharids (Hexoses) ($C_6H_{12}O_6$)	*Disaccharids* ($C_{12}H_{22}O_{11}$)	*Trisaccharids* ($C_{18}H_{32}O_{16}$)	*Polysaccharids* (Hexosans) ($C_6H_{10}O_5$)n
Glucose	Maltose	Raffinose	Starch
Fructose	Sucrose	Melicitose	Inulin
Galactose	Lactose		Dextrin
Mannose	Cellobiose		Glycogen
Sorbose	Melibiose		Galactan
			Cellulose
(Pentoses) ($C_5H_{10}O_5$)			(Pentosans) ($C_5H_8O_4$)n
Arabinose			Araban
Xylose			Xylan
Rhamnose ($C_6H_{12}O_5$)			

POLYHYDRIC ALCOHOLS	*GLUCOSIDES*
Mannitol	Salicin
Glycerol	Amygdalin
Adonitol	
Dulcitol	
Sorbitol	

EXERCISE 39

GELATIN LIQUEFACTION

Make two gelatin stab cultures each of *Streptococcus lactis, Bacterium coli, Bacillus subtilis,* and *Proteus vulgaris.* Incubate one culture of each organism at 20° C. and the other set at 37° C.; include with each set one sterile tube as a control.

After four days, examine the cultures incubated at 20° C. for liquefaction. Chill the tubes, incubated at 37° C., in cold water and observe whether the solidifying power of the gelatin has been destroyed. Care should be taken not to agitate the tubes incubated at 37° C. while in the liquid condition, as in cases of partial liquefaction the reaction would be obscured by the mixing of the liquefied portion with the unchanged gelatin.

EXERCISE 40

REACTIONS IN MILK

Using cultures of *Streptococcus lactis, Bacterium coli, Bacillus subtilis,* and *Proteus vulgaris,* inoculate tubes of:

(1) Plain milk
(2) Litmus milk
(3) Brom cresol purple milk

Incubate at 37° C. for four days and observe the changes which have taken place.

Plain Milk Medium

Plain milk medium consists simply of fresh skimmed milk. Fresh milk should be used; milk in which the acidity has begun to rise is apt to coagulate when sterilized.

Milk media may be sterilized in the autoclave or by the intermittent method. Milk, especially milk of poor quality which is heavily contaminated with bacteria, is frequently difficult to sterilize, hence the autoclave method is more sure. On the other hand, milk is browned somewhat at high temperatures due to the caramelization of the milk sugar.

Litmus Milk

Litmus milk consists of plain skimmed milk to which sufficient litmus solution has been added to impart a light purple color. For this purpose, a saturated aqueous solution of litmus should be used. Because of the great variation in the commercial litmus preparations, a definite rule as to amount necessary cannot be given.

Besides being of value to indicate the production of acidity or alkalinity in milk by bacteria, litmus is reduced by many bacteria to a colorless compound. The reduction of litmus is of some differential value in the study of bacteria.

After the litmus has been reduced it regains its color upon standing due to oxidation by the oxygen of the air.

Brom Cresol Purple Milk

Brom crésol purple is the laboratory name of Di-bromo-ortho-cresol-sulfonphtalein. As a substitute for litmus in milk media, brom cresol purple has the advantage of having a known chemical composition and is obtainable in the pure state. In the concentration used, it has little, if any, inhibitory effect upon the growth of bacteria. It is a more sensitive indicator for the reaction changes produced in milk by bacteria than is litmus. Brom cresol purple is not readily reduced by bacteria or by heat. This is an added advantage for most purposes, but from the standpoint of detecting the reducing power of bacteria it is a disadvantage, since brom cresol purple is not reduced as is litmus by bacterial action.

Brom Cresol Purple Solution

A stock solution for use in brom cresol purple milk is made as follows:

Grind in a glass mortar 0.5 gram of brom cresol purple; add to the fine powder 14 c.c. of a N/10 NaOH solution and stir well. Transfer to a flask. Wash the mortar with 75 c.c. of distilled water and pour the wash water into the same flask. Shake until solution is complete. Make up to 100 c.c. with distilled water.

Ten c.c. of this solution added to a liter of milk gives a concentration of 0.005 per cent.

Exercise 41

REDUCTION OF NITRATES

Many bacteria have the power of reducing nitrates to nitrites; a few are able to break down nitrates to gaseous nitrogen, either free nitrogen or oxides of nitrogen.

Inoculate Smith fermentation tubes containing nitrate broth with *Streptococcus lactis, Proteus vulgaris,* and *Pseudomonas pyocyaneus.* The composition of the nitrate broth is:

0.3% beef extract
1.0% peptone
1.0% KNO_3
Distilled water

Incubate two days. If gas is produced in any of the tubes, test as in Exercise 37. Test all of the cultures for nitrites, using Trommsdorf's reagent.

Test for Nitrites

Place 3 drops of Trommsdorf's solution in a depression on a spot plate. Add 1 or 2 drops of sulphuric acid (1:3). Remove a loopful of the solution to be

tested and touch to the surface of the reagent. A blue color indicates the presence of nitrites.

Preparation of Trommsdorf's Solution

(1) Add slowly, with constant stirring, a boiling solution of 20 grams of zinc chloride in 100 c.c. of distilled water to a mixture of 4 grams of starch in water. Continue heating until the starch is dissolved as much as possible, and the solution is nearly clear.

(2) Dilute with water and add 2 grams of zinc iodide (potassium iodide will do).

(3) Dilute to 1 liter and filter.

(4) Store in well-stoppered bottles in the dark.

EXERCISE 42

INDOL PRODUCTION

Inoculate a tube of Dunham's peptone solution with *Bacterium coli.* Incubate at 37° C. until the next period and then test for the presence of indol.

Indol is formed from tryptophane by the action of some bacteria. Tryptophane is an amino acid and hence occurs in peptone which is a digestion product of proteins. It is of course essential for this test that proteins which contain tryptophane be used in the making of the peptone. The bacteriological peptones now on the market contain tryptophane.

Dunham's Peptone Solution

1.0% peptone
0.5% sodium chloride
Distilled water

Test for Indol (Erlich Method)

The reagent is a 2 per cent solution of paradimethylaminobenzaldehyde in 95 per cent alcohol. One cubic centimeter of this reagent is added to the culture, then drop by drop concentrated hydrochloric acid is added until a red zone appears between the alcohol and peptone solution. Not more than 0.5 c.c. of the acid is required. On standing the zone deepens and widens. The red color is soluble in chloroform and the test may be confirmed by shaking the culture with chloroform to see if the pigment is extracted. If it proves soluble, the test is considered positive.

EXERCISE 43

HYDROLYSIS OF STARCH

Prepare starch agar plates and inoculate them by streaking with the cultures furnished. Incubate at 37° C. and test.

Starch agar is made by adding 0.2 per cent of water soluble starch to nutrient agar.

The diastatic action of the organisms is tested by flooding the surface of the starch agar plates with a saturated solution of iodine in 50 per cent alcohol. A clear zone around the area on which the organisms have grown indicates hydrolysis of the starch.

EXERCISE 44

RELATION OF FREE OXYGEN TO GROWTH

Melt tubes of meat infusion agar and hold for a few minutes at 100° C. to expel the free oxygen. Cool to a temperature of 42° to 45° C. and inoculate with cultures of *Bacterium coli, Bacillus subtilis,* and *Clostridium sporogenes.* Be careful not to agitate the tubes so as to incorporate unnecessary air in the agar. After inoculation, solidify the agar by placing the tubes in water below 40° C. Incubate at 37° C. for two days and observe the types of growth.

EXERCISE 45

PIGMENT PRODUCTION BY BACTERIA

Make glucose agar slope and plain nutrient agar slope cultures in duplicate from the chromogenic organisms furnished. After inoculation, incubate one culture of each organism on each medium at 20° C. and at 37° C. Observe the color and nature of pigments produced. Make observations after two and four days' incubation.

SECTION 9: ISOLATION AND PURIFICATION OF BACTERIAL CULTURES

EXERCISE 46

LOOP DILUTION METHOD

To isolate an organism so as to get it in a pure culture, free of all other types of bacteria, a transfer may be made from an isolated colony on an agar plate to an agar slope (or other suitable culture medium). Since each colony develops, as a rule, from one single cell, or a group of cells of the same species of organism hanging together, a subculture made from a colony growing well removed from the other colonies on the plate will usually contain only one kind of bacterium. In order to obtain the colonies of the various bacteria from a mixed culture it must be "plated out" on agar in the proper dilution. For this purpose what is known as the "loop dilution method" is commonly used.

Procedure

Label sterile Petri dishes number 1, 2, and 3. Melt three tubes of nutrient agar and cool them to 42° to 45° C. Label one tube number 1; one, number 2; and the remaining one, number 3. Transfer one loopful of the mixed culture to the agar tube number 1. Mix the organisms and agar thoroughly by rolling the tube between the palms of the hands. Transfer 1 loopful of the agar and organisms from tube number 1 to tube number 2. Mix thoroughly. Transfer 2 loopfuls from tube number 2 to tube number 3. Pour the agar in tube number 1 into Petri dish number 1, and the agar in tube number 2 into Petri dish number 2, and that of tube number 3 into Petri dish number 3. This gives a dilution of the organisms and enough scattered colonies on one of the plates so that the colonies may be studied in detail.

When the agar has thoroughly hardened, invert plates and incubate at 37° C. After two days make agar slope cultures from well-isolated colonies of different types.

EXERCISE 47

STREAK PLATE METHOD

While not such a satisfactory method, bacteria may be isolated by simply streaking the surface of an agar plate with a little of the material on a loop.

Make streak plates from the mixed culture furnished, as demonstrated. Note especially the precautions and details essential to this technique.

EXERCISE 48

DIFFERENTIAL PLATING METHODS

In the isolation of bacteria advantage may sometimes be taken of the physiological characteristics of the organisms in such a way that the colonies of the organisms sought may be recognized directly on the plates. For example, bacteria which ferment lactose with the formation of acid may be readily isolated by plating with lactose agar which contains some brom cresol purple as an indicator. Under these conditions the colonies of the lactose fermenting organisms would be surrounded by a yellow zone due to the acidity resulting from the lactose fermentation.

By the loop dilution method plate out a sample of milk with brom cresol purple lactose agar. Examine the plates for the presence of acid forming colonies after incubation at 37° C. for one and two days.

By the same method plate out a sample of soil, using casein agar. The casein agar may be made by adding a small amount of skimmed milk to ordinary nutrient agar. Bacteria which have the power of breaking down proteins will digest the casein around their colonies, making a clear zone around the colony in the opaque plate. Examine the plates for casein digesting bacteria after two and four days' incubation at 37° C.

SECTION 10: QUANTITATIVE BACTERIOLOGICAL ANALYSIS

EXERCISE 49

QUANTITATIVE PLATING METHODS

Samples "X" and "Y" which are furnished represent two samples of water. Sample "X" is very pure water with a small number of bacteria. Sample "Y" is a sample of impure water which contains a large number of bacteria.

Melt the tubes of agar in flowing steam or in boiling water. Place the melted agar into a water bath at about 45°C. This should be done in time so that the agar will be cooled to the proper temperature when it is ready to be poured into the plates. When poured, the agar should be between 42° and 45° C.

With a sterile pipette place 1 c.c. of sample "X" into a sterile Petri dish. Mark this plate "X-1". With the same pipette place 1/10 c.c. of the same sample into another sterile Petri dish. Mark this plate "X-1/10".

Materials which contain a large number of bacteria cannot be "plated out" directly as was done with sample "X", but must be diluted with sterile water.

With a sterile pipette remove 1 c.c. of sample "Y" and place it into a 99 c.c. water blank. This bottle now contains 1 c.c. of the original sample diluted 100 times; thus 1 c.c. of this dilution is equivalent to 1/100 c.c. of the original sample. Shake this bottle vigorously 25 times to insure a uniform mixing of the sample, and to break apart bacteria which may be hanging together in clumps.

With another sterile pipette remove 1 c.c. from this bottle (the 1/100 dilution) and place it into a sterile Petri dish. Mark this dish "Y-1/100". With the same pipette place 1/10 c.c. of the 1/100 dilution into another Petri dish. Mark this plate "Y-1/1,000". With the same pipette take another 1 c.c. amount from this bottle and place it into another 99 c.c. water blank.

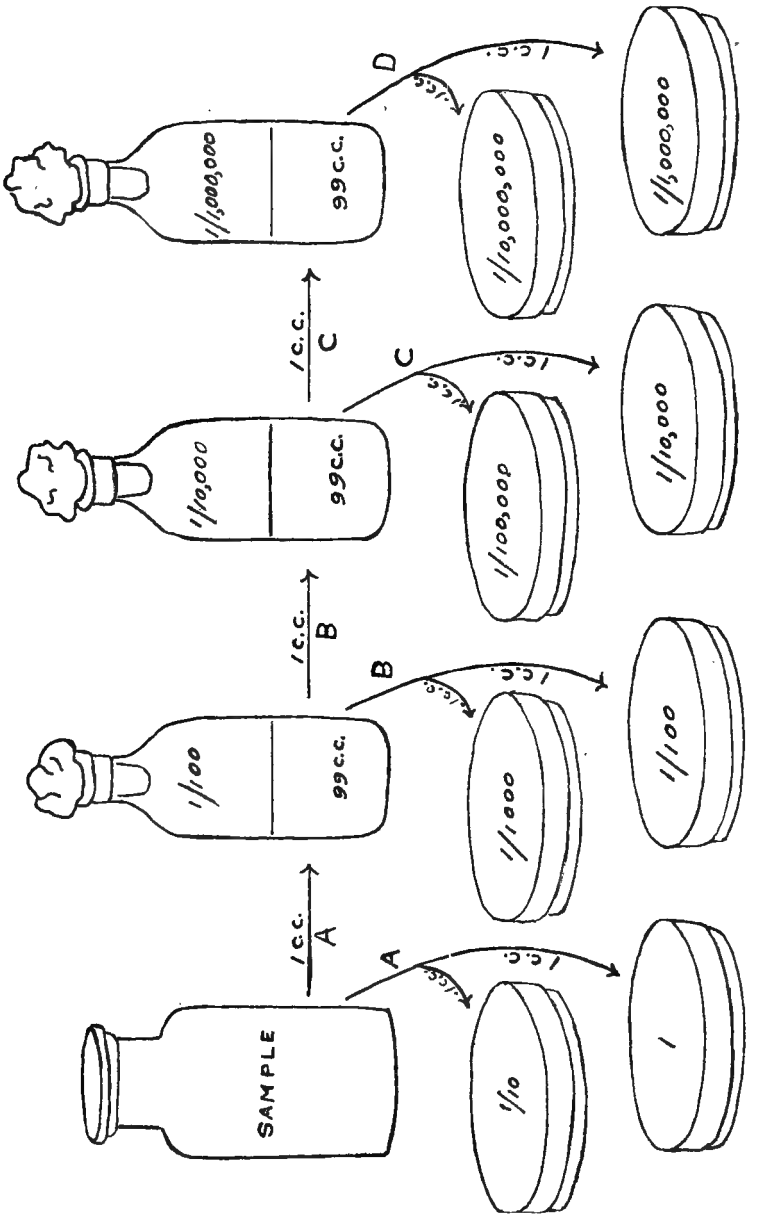
The second 99 c.c. water blank represents a 1/10,000 dilution of the original sample. Shake the second 99 c.c. water blank 25 times, and with another sterile pipette transfer 1 c.c. of it to a Petri dish. Label this dish "Y-1/10,000".

Pour the melted and cooled agar into the Petri dishes and mix thoroughly. Note especially the details of the technique involved as demonstrated.

In pouring the melted agar into the plates observe all the precautions outlined in Exercise 18. Be sure the temperature of the agar at the time of pouring is between 42° and 45° C. Thoroughly mix the melted agar and the dilution water in the plate by tilting and rotating the dish. In mixing, take especial care not to splash the agar on the sides and cover of the Petri dish. The mixing of the agar and the dilution water should be done immediately after the agar is poured into the dish before it has time to cool and harden.

When thoroughly solidified, invert the plates and incubate at 37° C. for two days.

It will simplify matters to arrange your Petri plates and water blanks at the beginning. Mark the plates and water blanks with their proper dilution figures. Also label the Petri dishes with such other marks of identification as may be needed. Make the dilutions desired in the manner outlined in the diagram given below. Be sure to change pipettes with each dilution bottle. On the diagram, the transfers which may be made with the same pipette are marked with the same letter.


SAMPLE
1 c.c.
A
99 c.c.
1/100
1 c.c.
B
99 c.c.
1/10,000
1 c.c.
C
99 c.c.
1/1,000,000
D
1/10
1
1/100
1/10,000
1/1,000,000
1/100,000,000
1/100
1/10,000
1/1,000,000
1/100,000,000

SECTION 11: ANAEROBIC METHODS

EXERCISE 50

ANAEROBIC CULTURE OF BACTERIA

With the culture of Clostridium welchii inoculate:

(1) A glucose infusion agar "shake" (Liborius' method)
(2) A tube of cooked meat or brain medium
(3) A glucose agar slope (for Wright's method)
(4) A glucose agar slope (for control)

Melt a tube of glucose infusion agar in boiling water or in flowing steam. The heating should be continued for about ten minutes so as to free the medium entirely of free oxygen. Cool in the water bath to 45° C. and inoculate. Take care not to shake the melted agar or to stir in air in making the inoculation. Place the inoculated tube in cold water to harden.

Heat a tube of cooked meat or brain medium in the same manner and cool to a temperature of 45° C. or below before inoculating.

Inoculate two glucose infusion agar slopes. Incubate one of those under aerobic conditions in the usual way as a control. With the other tube, push down the cotton plug about one-half inch below the top of the tube. Fill in the space above the cotton with pyrogallol. Pour upon the pyrogallol a small amount of concentrated NaOH and close tightly with a rubber stopper. Invert the tube and incubate in this position.

Incubate all the cultures at 37° C. Make observations at the next laboratory period.

SECTION 12: VARIABILITY OF BACTERIA AND RELATED PHENOMENA

EXERCISE 51

DISSOCIATION OF BACTERIA

By "bacterial dissociation" is meant the appearance of two or more variant forms from the same species. This phenomenon may be manifested by smooth ("s") and rough ("r") colonies; by slimy and non-slimy colonies; by motile and non-motile cells; and in other ways.

Inoculate a culture of *Bacterium aerogenes* into beef infusion broth having a pH of 7.8. Incubate for two weeks at room temperature.

After incubation, plate the culture on nutrient agar. At the same time plate as a control a culture of the same organism which has not been held in an alkaline medium. Incubate the plates at room temperature for three days.

Examine the plates for different types of colonies.

EXERCISE 52

FILTERABLE FORMS OF BACTERIA

It now appears to be fairly well established that some, and perhaps all, species of bacteria undergo a life cycle which includes forms small enough to pass through fine earthenware filters which remove bacteria of ordinary size. The "filterable forms" of bacteria appear to be quite different in their morphological, cultural and physiological properties from the ordinary forms of the same species.

Filter a broth culture of *Bacterium aerogenes* through a Berkefeld filter. Inoculate two glucose-beef infusion broth tubes with 1 c.c. each of the filtrate. Incubate at 37° C. for one day and then continue the incubation at 30° C. for two weeks. Also incubate one sterile tube of broth for a control.

After incubation, examine the tubes for the absence of ordinary bacterial growth. From one tube, place about two drops of the broth on the surface of each of two glucose-infusion agar plates. Add about 0.5 c.c. of sterile glucose-infusion broth to each plate and spread over the surface of the agar. Incubate the plates at 37° C. for two days. Make a control plate in the same way from the control tube of sterile broth.

After incubation, examine the plates for the presence of barely visible surface colonies. Make stained preparations with gentian violet from the surface growth and examine microscopically for the presence of definite cells.

EXERCISE 53

BACTERIOPHAGE

Bacteriophage is an ultramicroscopic, filterable substance which causes a lysis of bacterial cells.

Put two drops of bacteriophage filtrate on the surface of a four hours' old agar slope culture of *Bacterium aerogenes*. Drop the filtrate on the top of the slope so that it will run down over the bacterial growth. Treat in the same manner, for a control, another culture with two drops of sterile broth.

Keep at laboratory temperature and observe during the laboratory period.

SECTION 13: CLASSIFICATION OF BACTERIA

EXERCISE 54

THE DETAILED STUDY OF BACTERIA

Make a detailed study of the organisms furnished according to the Descriptive Chart supplied.

EXERCISE 55

IDENTIFICATION OF BACTERIA

Study the unknown cultures furnished in the same manner as was done in Exercise 54. From the facts regarding their characteristics, learned from this study, place them in their proper genera, according to the classification of Buchanan.

PART II

APPLIED BACTERIOLOGY

SECTION 14: THE QUANTITATIVE ESTIMATION OF MICROORGANISMS IN SOIL

EXERCISE 56

THE NUMBER OF BACTERIA IN SOIL

Two samples of soil are furnished. Sample No. 1 is a rich garden soil, while No. 2 is a sandy loam. In determining the number of bacteria in a solid substance, such as soil, the number is calculated per gram.

Weigh out samples of the soils furnished and make dilutions of 1/10,000; 1/100,000; and 1/1,000,000. Make duplicate plates from each of these dilutions with each soil. Melt twelve tubes of agar medium; when cooled to 42-45° C., pour the agar into the plates and mix with the dilution water. Incubate the plates at 30° C. for 4 days.

Count the number of colonies on your plates and calculate the number per gram in the original soil. Record in your note book the dilutions used, the number of colonies per plate, and the number of bacteria per gram of the original soil.

EXERCISE 57

THE NUMBER OF MOLDS IN SOIL

Plate the same samples of soil used in Exercise 56 in dilutions of 1/100; 1/1,000; and 1/10,000.

Use a special agar which is favorable for the growth of molds but which inhibits the development of bacteria. This may be accomplished by adjusting the reaction of the medium to a point too acid for the growth of the bacteria. The following medium is a good one for this purpose:

Glucose	1.0 per cent
Peptone	0.5 per cent
KH_2PO_4	0.1 per cent
$MgSO_4$	0.05 per cent
Agar	2.0 per cent

After sterilizing the above medium adjust to pH 4.0 by adding H_2SO_4.

Incubate the plates at 30° C. After incubation, count the colonies and calculate the number of molds per gram of soil.

SECTION 15: THE NITROGEN CYCLE IN NATURE

EXERCISE 58

AMMONIFICATION

Treat five tubes of a one per cent peptone solution as follows:

(1) Leave one tube as a sterile control.
(2) Inoculate with a loopful of soil.
(3) Inoculate with *Bacillus mycoides.*
(4) Inoculate with *Pseudomonas fluorescens.*
(5) Inoculate with *Proteus vulgaris.*

Incubate these tubes at 30° C.

At the end of 48 hours test the tubes for the presence of ammonia. Place a drop of Nessler's reagent on a spot plate; touch this with a loopful of the substance under test. The presence of ammonia is indicated by a yellow color.

Nessler's Reagent

To 50 grams of potassium iodide dissolved in distilled water add a saturated solution of mercuric chloride until a slight precipitate remains. Add 400 c.c of a 50 per cent solution of sodium hydroxide which has been clarified by sedimentation. Dilute to 1000 c.c. by adding distilled water, and allow to settle for one week before using. Decant the clear solution and keep in well stoppered bottles away from light.

EXERCISE 59

NITRIFICATION: NITRITE FORMATION

Inoculate a flask of the medium furnished with about 0.1 gram of soil. Incubate at laboratory temperature.

The medium used for this purpose has the following composition:

Ammonium sulphate $(NH_4)_2\ SO_4$	2.0 grams
Dibasic potassium phosphate (K_2HPO_4)	1.0 gram
Magnesium sulphate $(Mg\ SO_4)$	0.5 gram
Ferrous sulphate $(Fe\ SO_4)$	0.4 gram
Sodium chloride (NaCl)	0.4 gram
Water	1000.0 grams
Magnesium carbonate $(Mg\ Co_3)$	Excess

Test the culture every week for ammonia and nitrites. A test for nitrites should appear after a week or two and after several weeks the test for ammonia should fail owing to the complete oxidation of the ammonia present to nitrites.

The test for ammonia may be made with Nessler's solution by placing a drop of the solution on a spot plate and then touching this with a loopful of the culture.

Test for nitrites by mixing on a spot plate three drops of Trommsdorf's solution and two drops of sulphuric acid solution (1 part concentrated sulphuric acid to 3 parts water). Touch with a loopful of the culture.

EXERCISE 60

NITRIFICATION: NITRATE FORMATION

Inoculate a flask of the medium furnished with about 0.1 gram of soil and incubate at laboratory temperature.

The composition of this medium is as follows.

Sodium nitrite ($Na\ NO_2$)	1.0 gram
Dibasic potassium phosphate (K_2HPO_4)	1.0 gram
Magnesium sulphate ($Mg\ SO_4$)	0.3 gram
Sodium carbonate (Na_2CO_3)	1.0 gram
Sodium chloride ($NaCl$)	0.5 gram
Ferrous sulphate ($Fe\ SO_4$)	0.4 gram
Water	1000.0 grams

Test the flask each week for nitrites until the test fails due to the complete oxidation to nitrates. When a test is no longer obtained for nitrites test the culture for nitrates. Since the test for nitrates also reacts for nitrites it can only be used in the absence of nitrites.

The test for nitrates is made as follows: On a spot plate put one drop of diphenylamine reagent and two drops of concentrated sulphuric acid. Touch with a loopful of the culture. A positive test for nitrates will be shown by a dark blue color.

Diphenylamine reagent

To 28.8 c.c. of distilled water add 60 c.c. of concentrated sulphuric acid and 0.7 gram of diphenylamine. Allow the solution to cool. Add slowly 11.3 c.c. of concentrated hydrochloric acid.

EXERCISE 61

DENITRIFICATION

Inoculate a fermentation tube of nitrate broth with about 0.1 gram of soil. Inoculate another tube with a pure culture of *Pseudomonas pyocyaneous*. Incubate at 30° C. The nitrate broth is the same as ordinary nutrient broth with the addition of one per cent of $Na\ NO_3$.

Determine the composition of the gas produced. If the gas is not absorbed by NaOH and does not form an explosive mixture with air, it is probably nitrogen.

EXERCISE 62

NON-SYMBIOTIC NITROGEN FIXATION

The bacteria which fix free nitrogen from the air may be grown on a medium which contains no nitrogen, only inorganic salts, and a simple carbohydrate from which they derive energy. The medium is composed of the following ingredients:

Mannitol [C_6H_8 $(OH)_6$]	15.0 grams
Diabasic potassium phosphate (K_2H PO_4)	0.2 gram
Magnesium sulphate (Mg SO_4)	0.2 gram
Sodium chloride (NaCl)	0.2 gram
Calcium sulphate (Ca SO_4)	0.1 gram
Calcium carbonate (Ca CO_3)	5.0 grams
Water	1000.0 grams

Inoculate a flask containing a shallow layer of the above medium with about one gram of a fertile soil. Incubate at 30° C.

Examine a mannitol agar slope (the above medium plus 1.5 per cent agar) of *Azotobacter chroococcum* and note the characteristics of the growth. Make microscopic preparations of the organism and stain. Note carefully its morphology and make drawing.

When the flask containing the nitrogen-free mannitol medium shows a heavy growth, make stained microscopic preparations from the surface growth. Examine for large oval or coccoid cells of *Azotobacter*.

EXERCISE 63

SYMBIOTIC NITROGEN FIXATION

Examine the plant furnished and observe the position of the nodules on the roots.

Select a nodule from your plant and carefully wash it in clean water. Grind the nodule with a drop of water on a clean slide. From this suspension make a smear preparation on another slide. Stain and examine under the oil-immersion objective. Look especially for irregularly shaped cells; such as, pear, club, "Y" and "T" shaped individuals. The bacteria when appearing in these shapes are called "bacteriods". Draw the preparation.

The legume bacteria may be grown in the laboratory on a medium similar to that used for the cultivation of the independent nitrogen fixing bacteria. These organisms when grown on artificial culture media do not appear as irregularly shaped bacteriods, but as ordinary rod-shaped organisms.

Note the type of growth produced by *Rhizobium leguminosarum* on the agar slope furnished and make a stained preparation from it. The legume organism when taken from artificial media does not stain easily; usually the slimy growth material will take the stain, leaving the rods unstained. The bacteria thus appear as colorless bodies on a stained background.

SECTION 16: BACTERIOLOGY OF AIR

EXERCISE 64

THE RELATIVE NUMBERS OF BACTERIA IN AIR

Pour four tubes of nutrient agar into sterile Petri dishes and allow to harden.

Expose duplicate plates as follows:

(1) To the air of the laboratory for 5 minutes.

(2) To the air of the laboratory for 20 minutes.

Invert the plates and incubate either at 37° C. for 2 days or at room temperature for 4 to 6 days. Count the number of colonies which develop but do not open the plates.

Observe the types of colonies which develop. Make Gram stains and study microscopically.

EXERCISE 65

THE QUANTITATIVE BACTERIAL EXAMINATION OF AIR

Draw ten liters of the laboratory air through a tube containing 5 c.c. of sterile water.

Plate the sample from the laboratory air in duplicate, using 1 c.c. amounts. Incubate the plates at 37° C. for 2 days, or at room temperature for 4 to 6 days.

Tabulate the data obtained, recording the results in terms of the number of bacteria per liter and per cu. ft. of air.

SECTION 17: BACTERIOLOGY OF WATER

EXERCISE 66

STUDY OF THE COLI-AEROGENES GROUP OF BACTERIA

Make a detailed study of cultures representing different types of the coli-aerogenes group. Determine: (1) morphology, (2) staining reactions, (3) spore formation, (4) cultural characteristics on agar slopes, (5) gelatin liquefaction, (6) indol production, (7) milk reactions, (8) type of colony on brom cresol purple lactose agar, (9) fermentation reactions in glucose, lactose, and sucrose broths, (10) methyl red test, (11) Voges-Proskauer reaction.

The Methyl Red Test

Grow the cultures in a medium containing:

0.5% glucose
0.5% peptone
0.5% dibasic potassium phosphate

Incubate for 5 days at 30° C. (37° C. for 2 days may be used).

Determine the resulting hydrogen ion concentration using methyl red as the indicator. Cultures which are acid to this indicator are designated positive to the methyl red test.

The Voges-Proskauer Reaction

This test is based upon the ability of certain organisms to produce acetyl-methyl-carbinol from glucose. For this purpose part of the culture which is used for the methyl red test may be utilized. (Ordinary glucose broth could be used for the Vosges-Proskauer reaction, but not for the methyl red test.)

After incubation, test as follows: to 5 c.c. of the glucose broth culture, add 5 c.c. of a 10% solution of KOH. Allow to stand over night. A positive test for acetyl-methyl-carbinol is indicated by the presence of an eosin pink color.

Another test for the presence of acetyl-methyl-carbinol, which gives quicker results, may be used. Add 5 c.c. of a 10% solution of KOH to 1 to 4 c.c. of a 48-hour culture in methyl red media (or glucose broth). Boil the culture for a few minutes by holding the tube in boiling water. The reaction may be hastened by shaking the tube.

EXERCISE 67

EXAMINATION OF WHOLESOME WATER

Obtain some water by allowing the faucet to run at least ten minutes before collecting the sample in a sterile flask.

Make duplicate plates of this sample using 1 c.c. and 1/10 c.c. amounts. Make one plate from each dilution with lactose agar and the other with either litmus lactose agar or brom cresol purple lactose agar.

Inoculate lactose broth fermentation tubes with 10 c.c., 1 c.c., and 1/10 c.c. amounts of the same water.

Incubate the plates and fermentation tubes at 37° C. for 48 hours. If possible, also observe the results at the end of 24 hours. Determine the bacterial count of the water from the lactose agar plates. Determine the presence or absence of acid-forming colonies from the lactose agar plates containing indicator. Observe the fermentation tubes for gas formation; if present, analyze the gas.

Exercise 68

EXAMINATION OF SEWAGE

Plate out a sample of sewage using dilutions of 1/10,000, 1/100,000, 1/1,000,000, and 1/10,000,000. Make duplicate plates using lactose agar and either litmus lactose agar or brom cresol purple lactose agar.

Inoculate lactose broth fermentation tubes using the same dilution as used in the plates.

Incubate at 37° C. and record results as in the previous exercise.

Exercise 69

EXAMINATION OF SEWAGE POLLUTED WATER

Examine a sample of polluted water in the same way as outlined in the previous exercises, using dilutions of 1/10, 1/100, and 1/1,000 for plating. Inoculate lactose broth fermentation tubes with the same dilutions as used in the plates.

Incubate and observe as before.

Exercise 70

EXAMINATION OF UNKNOWN WATERS

Examine samples of unknown waters using the same methods employed in the previous exercises. For plating employ 1 c.c., 1/10 c.c., and 1/100 c.c. amounts. Inoculate the fermentation tubes with 10 c.c., 1 c.c., and 1/10 c.c. amounts.

EXERCISE 71

CONFIRMATORY TESTS FOR BACTERIUM COLI IN WATER

Isolate cultures from acid producing colonies found in some of the samples of water which have given presumptive tests for organisms of the coli-aerogenes group.

Make further studies to confirm these cultures as belonging to the coli-aerogenes group. Determine also whether they belong to the coli or aerogenes division of the group, and their probable species.

Determine: (1) morphology, (2) staining reactions, (3) spore formation, (4) cultural characteristics on agar slopes, (5) gelatin liquefaction, (6) indol production, (7) milk reactions, (8) methyl red test, (9) Voges-Proskauer reaction, (10) fermentation reactions in glucose, lactose, and sucrose broths.

EXERCISE 72

LACTOSE PEPTONE BILE MEDIUM

Inoculate Smith fermentation tubes containing lactose peptone bile with cultures of *Bacterium coli* and *Bacterium aerogenes* and with 1/10 c.c. of a sample of polluted water. Incubate at 37° C.

The composition of the lactose peptone bile used for this purpose is:

Lactose	1.0%
Peptone	1.0%
Oxgall (dried)	5.0%

Five per cent of dried oxgall gives a solution of about one-half the strength of the bile as it comes from the gall bladder.

The principle underlying the use of bile in the examination of water is that it inhibits the growth of many bacteria not of intestinal origin, while inhibiting only slightly or not at all the intestinal types.

EXERCISE 73

ENDO MEDIUM

By the loop dilution method, plate out cultures of *Bacterium coli* and *Bacterium aerogenes* on Endo's agar. Incubate at 37° C.

The composition of Endo medium is:

Dibasic potassium phosphate	0.35%
Peptone	1.0%
Agar (washed)	1.5%
Lactose	1.0%

To the above, just before using, add:

Sodium sulphite (anhydrous)	0.25%
Basic fuchsin (10% alcoholic solution)	0.5%

In this medium, when properly prepared, the sodium sulphite solution decolorizes the intense red of the fuchsin to a light pink. Organisms of the coli-aerogenes type change the medium to a bright red color and their colonies usually show a metallic luster. The coli types, as a rule, produce a more pronounced metallic luster than do the aerogenes types.

EXERCISE 74

EOSINE-METHYLENE BLUE AGAR

In the same manner as with Endo medium, plate the same cultures on eosine-methylene blue agar and incubate at 37° C.

The composition of this medium is:

Dibasic potassium phosphate	0.2%
Peptone	1.0%
Agar	1.5%
Lactose	1.0%
Eosine	0.04%
Methylene blue	0.01%

By the use of this medium it is possible, with practice, to differentiate bacteria of the coli type from those of the aerogenes type. While this medium does not positively differentiate these organisms, one experienced in its use can differentiate between these types in about 90% of the cases.

DIFFERENTIATION OF BACTERIUM COLI AND BACTERIUM AEROGENES ON EOSINE-METHYLENE BLUE AGAR

	(1) *Bacterium coli*	(2) *Bacterium aerogenes*
Size	Well isolated colonies are 2-3 m.m. in diameter.	Well isolated colonies are larger than coli; usually 4-6 m.m. in diameter or more.
Confluence	Neighboring colonies show little tendency to run together.	Neighboring colonies run together quickly.
Elevation	Colonies slightly raised; surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance by Transmitted Light	Dark, almost black centers which extend more than ¾ across the diameter colony; internal structure of central dark portion difficult to discern.	Centers deep brown; not as dark as *Bacterium coli* and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies.
Appearance by Reflected Light	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen.	Much lighter than *Bacterium coli,* metallic sheen not observed except occasionally in depressed center when such is present.

(1) *Bacterium coli.* Two other types have been occasionally encountered: One resembles the type described, except that there is no metallic sheen, the colonies being wine colored. The other type of colony is somewhat larger (4 m.m.), grows effusely, and has a marked crenated or irregular edge, the central portion showing a very distinct metallic sheen. These two varieties constitute about 2 or 3 per cent of the colonies observed.

(2) *Bacterium aerogenes.* A small type of aerogenes colony, about the size of the colon colonies, which show no tendency to coalesce, has been occasionally encountered.

SECTION 18: BACTERIOLOGY OF MILK

EXERCISE 75

THE PLATE CULTURE METHOD FOR THE QUANTITATIVE BACTERIOLOGICAL ANALYSIS OF MILK

Plate out a sample of high grade milk in dilutions of 1/100 and 1/1,000. Plate a sample of low grade milk in dilutions of 1/10,000 and 1/100,000. Use lactose agar as the plating medium. Incubate the plates at 37° C. for two days.

After incubation, count the colonies and estimate the number of bacteria per c.c. of milk in each case.

EXERCISE 76

DIRECT MICROSCOPIC DETERMINATION OF BACTERIA IN MILK

(1) By means of a stage micrometer, using the oil immersion objective, adjust the tube length of your microscope so that the microscopic field has a diameter of 0.16 m.m.

(2) Spread 0.01 c.c. of milk over exactly 1 sq. cm. area on a microscopic slide. Make duplicate smears on the same slide.

(3) Dry the films of milk very slowly on a perfectly level surface. Extreme caution must be taken if heat is applied in drying.

(4) Immerse the slide in xylol for about 1 minute to remove fat. Dry.

(5) When dry, immerse in 95% alcohol to fix to slide.

(6) Stain by immersion in methylene blue (1.0% methylene blue in 50% alcohol) until the film has a definite blue color. Avoid over-staining.

(7) Wash the slide with water.

(8) In case the preparation is stained too deeply, it may be decolorized to a light blue by immersion in 95% alcohol. When this step is taken, care must be used not to decolorize the bacteria.

(9) By use of the oil immersion objective, determine the number of bacteria per field. A number of fields must be counted to obtain accurate results. The greater the number of bacteria in the milk, the smaller the number of fields necessary to be counted in order to obtain accurate results. A good rule is to count 100 fields in the case of a high grade milk, and in the case of a low grade milk, 10 fields.

(10) From the counts obtained estimate the number of bacteria per c.c. of milk.

With 0.01 c.c. of milk spread over an area of 1 sq. cm., and with a microscopic field of 0.16 m.m. diameter, the number of bacteria per field multiplied by 500,000 gives the number of bacteria per c.c. of the original milk.

Derivation of the Factor for the Microscopic Count

Diameter of microscopic field=0.16 m.m.
πR^2=Area of microscopic field.
R =0.08 m.m.
R^2=0.0064
0.0064 × 3.1416=0.02 sq. m.m.—area of microscopic field
0.02 × 0.01=0.0002 (or 1/5,000) sq. cm.—area of microscopic field.

Since 1/100 c.c. of milk was spread over 1 sq. cm., each microscopic field covers 1/100 × 1/5,000 or 1/500,000 c.c. of milk. Therefore, each bacterium in a field represents 500,000 per c.c. of milk.

Total No. in 1 field × 500,000=No. per c.c.
Total No. in 10 fields × 50,000=No. per c.c.
Total No. in 50 fields × 10,000=No. per c.c.
Total No. in 100 fields × 5,000=No. per c.c.

EXERCISE 77

METHYLENE BLUE REDUCTION TEST

Using sterile pipettes, place 1 c.c. amounts of the methylene blue solution in sterile test tubes. Add 10 c.c. samples of the milks under test. See that they are thoroughly mixed.

The solution of methylene blues furnished is of such strength that the sample under test contains one part of the dry dye in 200,000 parts of the mixture.

Place the samples in a water bath at 37° C. and observe the time which elapses before the color disappears.

After the milk samples and dye are thoroughly mixed, the sample should not be agitated, which would allow the admixture of air which would retard the reduction of the methylene blue.

By the use of this test milks may be roughly divided into classes, as given below.

Bact. per c.c.	*Time of Reduction*
<50,000	>10 hrs.
<500,000	>5½ hrs.
500,000 to 4,000,000	>2 hrs. <5½ hrs.
4,000,000 to 20,000,000	>1/3 hr. <2 hrs
>20,000,000	<1/3 hr.

The Methylene Blue Solution

There are on the market special tablets of methylene blue of such strength that when one is dissolved in 200 c.c. of distilled water, and 1 c.c. of the solution is added to 10 c.c. of milk it gives approximately the above mentioned concentration (1 part of methylene blue to 200,000 parts of the mixture).

If tablets are not available, a stock solution may be prepared by dissolving

1.1 gms. of the dry dye (medicinal methylene blue) in 500 c.c. of distilled water. When needed, 1 c.c. of the stock solution is measured out and diluted with distilled water to 40 c.c. One c.c. of this diluted portion contains $\frac{55}{1{,}000{,}000}$ gms. of the dye. This amount added to 10 c.c. of milk gives a concentration of approximately $\frac{55}{11{,}000{,}000} = \frac{1}{200{,}000}$ in the final mixture.

The stock solution keeps for long periods due to the toxicity of methylene blue at that concentration. Nevertheless, it is advisable to heat the dilute solution to 100° C. before use.

EXERCISE 78

A STUDY OF STREPTOCOCCUS LACTIS

From the culture of *Streptococcus lactis* furnished, inoculate: agar slope; glucose, lactose and sucrose broth Durham tubes; gelatin stab; and plain milk, litmus milk, and brom cresol purple milk.

By the loop dilution method plate the culture on lactose agar in order to study the type of colony produced. Incubate the cultures and plates at 37° C. until the next period.

Make a Gram stain preparation from the agar slope culture and make drawing. Is it Gram positive? Does it produce spores?

Note: The type of growth produced on the agar slope; the type of colony produced on the Petri plates; which sugars are fermented, and whether gas is produced in the fermentation of sugars by this organism; and whether gelatin is liquefied.

From the brom cresol purple and litmus milk cultures, note reaction changes caused by the production of acid or alkali. Note whether litmus is reduced. Observe the plain milk tube for the presence of coagulation, type of curd produced, and whether there is any digestion of the casein. From this culture determine the amount of lactic acid produced in milk.

Determination of Lactic Acid in Milk

Take 5 c.c. of milk from the culture to be tested and dilute with 5 c.c. of distilled water. Using phenolphthalein as indicator, titrate with N/20 NaOH. Calculate the per cent of acid as lactic by use of the formula:

$$\text{Per cent as lactic acid} = \frac{\text{c.c. N/20 NaOH} \times .0045 \times 100}{\text{c.c. milk used}}$$

EXERCISE 79

STUDY OF BACTERIUM COLI AND BACTERIUM AEROGENES

Review the general morphological, cultural, and physiological characteristics of organisms of the coli-aerogenes group as determined in Exercise 66.

What action do the members of this group of organisms have upon lactose? Do they liquefy gelatin? From these characteristics, what action would you expect them to have on milk?

Inoculate tubes of plain milk, litmus milk, and brom cresol purple milk with *Bacterium coli* and *Bacterium aerogenes*. Also inoculate Smith fermentation tubes containing lactose broth with these organisms. Incubate all the cultures at 37° C. for two days.

Note carefully the type of fermentation produced in milk by these organisms: whether acid is produced, the milk curdled, type of curd produced, evidences of gas formation in the curd and in the fermentation tube, and whether there is any evidence of peptonization or casein digestion. Titrate a sample from the plain milk culture and calculate its acidity as lactic acid.

EXERCISE 80

STUDY OF LACTOBACILLUS BULGARICUS

From the milk culture of *Lactobacillus bulgaricus* furnished make stained preparations using methylene blue and Gram's stain. Study the morphology of the organism and make drawings. Note whether Gram positive or negative.

Inoculate: (1) nutrient agar slope, (2) plain nutrient broth, (3) litmus milk, (4) brom cresol purple milk, and (5) three tubes of plain milk. Incubate at 37° C.

Observe the agar slope and plain bouillon cultures. Is there much evidence of growth on these media? Observe the milk cultures for: acid production, coagulation of milk, type of curd produced, evidences of gas formation, and casein digestion. Titrate the plain milk cultures and calculate the percentage of lactic acid after 2, 7, and 14 days' incubation at 37° C.

Although members of the Lactobacillus group do not grow rapidly enough at low temperatures to take part in the ordinary souring of milk, they are always present in ordinary milk and will develop upon prolonged incubation. Fill a small bottle with skimmilk. The bottle should be filled to the neck and then tightly stoppered so as to prevent mold growth. Incubate at 37° C. Make microscopic preparations using methylene blue and Gram's stain after 2, 7, and 14 days' incubation. Make a drawing of a typical field at each of these periods. Determine the percentage of lactic acid produced after 2, 7, and 14 days' incubation at 37° C.

EXERCISE 81

STUDY OF CASEIN DIGESTING BACTERIA

Many bacteria are proteolytic in their metabolism rather than fermentative. Such organisms when grown in milk will peptonize or digest the casein instead of fermenting the lactose with the production of acid. Many different species of bacteria having this property are always present in milk but under normal conditions their activities are inhibited by the lactic acid bacteria. Hence, milk usually sours instead of undergoing a proteolytic decomposition.

From cultures of *Bacillus subtilis, Pseudomonas pyocyaneus* and *Proteus vulgaris* inoculate: (1) plain agar slopes, (2) gelatin stabs, (3) Durham tubes of brom cresol purple lactose broth, (4) plain milk, and (5) brom cresol purple milk.

After incubation make a study of their morphological and cultural characteristics. Which of these organisms produce spores? What was their action in the brom cresol purple lactose broth? Do they liquefy gelatin? From the results obtained with lactose broth and gelatin, what action would you expect these organisms to have on milk? What were the results obtained with the cultures grown in plain milk and brom cresol purple milk?

EXERCISE 82

PASTEURIZATION OF MILK

Obtain a sample of raw market milk and plate it out on lactose agar using dilutions of 1/10,000 and 1/100,000.

Pasteurize the milk by heating in water to 62.8° C. (145° F.) for 30 minutes. Plate out the pasteurized milk using dilutions of 1/100 and 1/1,000. Incubate all the plates at 37° C. for 48 hours.

Hold tubes of pasteurized and raw milks at laboratory temperature. Observe the length of time each sample remains sweet, and the type of fermentation which occurs in each. From the results obtained, is it your opinion that all of the lactic acid producing bacteria are destroyed at the temperatures used in pasteurization? Of what significance is this fact?

SECTION 19: BACTERIOLOGY OF DISEASE

Exercise 83

STUDY OF PYOGENIC STAPHYLOCOCCI

A number of pyogenic (pus producing) infections, such as boils, carbuncles, abscesses, etc., are caused by staphylococci. The most important of these are *Staphylococcus albus* and *Staphylococcus aureus*.

From cultures of *Staphylococcus albus* and *Staphylococcus aureus* inoculate: (1) agar slope, (2) nutrient broth, (3) gelatin stab, (4) brom cresol purple glucose, lactose, and sucrose broths in Durham tubes, and (5) litmus milk and brom cresol purple milk.

Incubate all cultures, except the gelatin, at 37° C.

Make morphological studies using Gram's stain.

Exercise 84

STUDY OF STREPTOCOCCUS PYOGENES

Streptococcus pyogenes is the common cause of pyogenic infections, such as boils, carbuncles, and abscesses; wound infections; inflammation of mucous membranes, such as tonsilitis; and infections of internal organs, as in certain cases of appendicitis and endocarditis. Other closely related streptococci are responsible for a number of diseases in man and animals; some examples of which are *Streptococcus scarlatinae,* cause of scarlet fever; *Streptococcus erysipelatis,* the cause of erysipelas; *Streptococcus equi,* the cause of strangles in horses; *Streptococcus mastitidis,* the cause of inflammation of the udder or garget in cattle.

From the culture of *Streptococcus pyogenes* inoculate: (1) agar slope, (2) nutrient broth, (3) gelatin stab, (4) brom cresol purple glucose, lactose, and sucrose broths in Durham tubes, and (5) litmus milk and brom cresol purple milk.

Incubate all cultures, except the gelatin, at 37° C.

Make morphological studies using Gram's stain.

Exercise 85

STUDY OF CORYNABACTERIUM DIPHTHERIA

From a culture of the diphtheria organism make stained microscopic preparations, using methylene blue. Study carefully and observe for the presence of cells showing irregular staining and variations in morphology.

EXERCISE 86

THE COLON-TYPHOID BACTERIA

In the general class of the intestinal or colon-typhoid bacteria are included three divisions.

(1) The coli-aerogenes group, includes *Bacterium coli, Bacterium aerogenes* and a number of other species which have been previously studied in connection with water and milk. These organisms are non-pathogenic, although *Bacterium coli* is occasionally associated with certain troubles, especially inflammation of the urinary and gall bladder.

(2) The enteritidis-paratyphoid group includes a number of closely related species. *Bacterium enteritidis* and *Bacterium paratyphi* (types A and B) are found especially as the causes of meat poisoning and are also spread to some extent by polluted milk and water. In this group belong a number of other very closely related species which are responsible for diseases in lower animals. The more important of these are: *Bacterium suipestifer,* a secondary invader in hog cholera; *Bacterium pollorum,* the cause of white diarrhea in chicks, and *Bacterium typhi-murium,* the cause of mouse typhoid.

(3) The typhoid-dysentery group includes the organism of typhoid fever, *Bacterium typhosum,* and the casual organism of bacterial dysentery in man, *Bacterium dysenteriae.*

Study cultures of *Bacterium coli, Bacterium aerogenes, Bacterium enteritidis, Bacterium paratyphi, Bacterium typhosum,* and *Bacterium dysenteriae* as follows: (1) Morphological study including motility and reaction to the Gram stain; (2) action on gelatin; (3) action on litmus milk; (4) fermentative characteristics in glucose and lactose broths in Durham tubes.

From this study, what morphological and cultural characteristics appear to be common to all of the colon-typhoid organisms? According to the results obtained in glucose and lactose broths and litmus milk, what differences are found between the three divisions of the colon-typhoid organisms?

EXERCISE 87

TUBERCULOSIS

Study the type of growth produced by *Mycobacterium tuberculosis* on the agar slope culture furnished. The agar used for this purpose is beef infusion agar with the addition of five per cent glycerol. Make a stained preparation using the acid-fast staining method outlined in Exercise 19, and study its morphology.

Examine the samples of sputa supplied for the presence of the organism of tuberculosis, employing the method previously used (Exercise 19).

Make a post-mortem examination of a tubercular guinea pig. Note the locations and characteristics of the lesions. Make slide preparations from typical

lesions. Apply the acid fast stain and examine for the presence of *Mycobacterium tuberculosis.*

EXERCISE 88

ANTHRAX

From the culture of *Bacillus anthracis* furnished, make cultures on (1) agar slope, (2) nutrient broth, (3) gelatin and (4) litmus milk. For comparative purposes, inoculate additional tubes of the same media with *Bacillus cereus.* Incubate all the cultures at 37° C.

Note the cultural characteristics of these organisms on the different media. Make stained preparations by the Gram method from the agar slope and broth cultures. Note the morphology and staining characteristics of the organisms. In which media is spore formation more abundant? How do these organisms compare in morphological and cultural characteristics, and in motility?

Observe the subcutaneous inoculation of a guinea pig with *Bacillus anthracis.* Note carefully the details of the inoculation as demonstrated. As a control, inoculate another guinea pig with an equal amount of *Bacillus cereus.*

Immediately after death of the animal inoculated with the anthrax bacillus make a post-morten examination. Note the condition of the various internal organs. Make agar slope cultures from the spleen, liver and heart blood and incubate at 37° C. Make Gram stain preparations from the spleen, liver and heart blood. After incubation, examine the agar slope cultures made from organs of the anthrax infected guinea pig. Note whether the type of growth and morphology are typical of *Bacillus anthracis.*

Note if any ill effects have been produced in the guinea pig inoculated with *Bacillus cereus.*