Laboratory Manual
of
General Agricultural
Bacteriology

Hastings and Wright

Class QR51

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A Laboratory Manual

of

General Agricultural Bacteriology

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Preface to the First Edition

With the present day tendency in our agricultural colleges to crowd much into the limitations of the curriculum, the problem of what to give and what not to give in a general course becomes a perplexing one.

More than ten years of experience teaching agricultural, home economics, science and engineering students have contributed to the development of the present edition of the Laboratory Manual of General Agricultural Bacteriology. Many of the changes that have been made from time to time have come as the result of class room experience and the need for more economical utilization of the time of both instructor and student.

No attempt is made to cover completely and in detail all phases of morphology, cultural characters and physiology of bacteria. Enough of each of these is included in connection with practical exercises to give the student a general conception of the relation of bacteria to agricultural processes. For those who wish more detailed information the advanced courses are available; while the students going into other lines of agriculture taking it as a required course find themselves broadened by a general knowledge of the relation of bacteria to practical things.

The exercises as outlined have been planned for local use. No effort has been made to arrange them for all kinds of conditions. On this account their use elsewhere may require some slight modifications. The number and variety of the exercises offer considerable latitude in the choice of work for a course. This is desirable where the type of the students in the course changes from semester to semester.

THE AUTHORS.

Preface to the Second Edition

The reception of the first edition of the Laboratory Manual of General Agricultural Bacteriology has been very satisfactory. The issuing of a second edition, with such changes as may be considered necessary, is primarily for the needs of the authors' classes.

The plan of the book has not been materially changed, but more detail has been introduced into several of the exercises. These changes are considered desirable because the laboratory time of the student has been increased considerably.

It is hoped that others who have been using the manual will find the changes equally acceptable and adapted to their needs.

University of Wisconsin
September, 1924.

THE AUTHORS.

PART I.

GENERAL DIRECTIONS LABORATORY REGULATIONS

1. **Desks and Lockers.** Each student is assigned a desk and given an individual locker in this desk. The student will be held responsible for the condition of the desk at the end of each meeting of his laboratory section.

2. **Apparatus.** The apparatus needed in the laboratory work will be found in the locker. The apparatus should be checked with the list placed on the blackboard by the instructor. Missing articles should be obtained from the storeroom before the locker is accepted. Any damaged article should be exchanged at the storeroom.

3. **Supplies.** The materials needed for the work of each laboratory period will be placed on the desk before the section assembles. Additional supplies, which may be needed from time to time, may be secured at the storeroom. On one of the **white** cards to be found in the metal box on the desk, place your name, locker number, and the supplies you wish. Present the card thus made out to the storeroom.

4. **Return of Apparatus.** On one of the **blue** cards to be found in the metal box on the desk, place your name, locker number, and list of the apparatus you are to return. Present the card, together with the apparatus, at the storeroom.

All glassware returned to the storeroom must be **clean** and **dry**. Apparatus is to be returned at such times and in such amounts as may be announced.

5. **Waste Material.** All waste paper, cotton plugs, matches, and broken glassware must be placed in the central compartment of the metal box to be found on each desk.

6. **Washing Apparatus.** All dirty apparatus is to be washed at the large wall sinks. The small sinks are for staining purposes only.

7. **Arrangement of Desks.** At the end of each laboratory period each student must place his desk in order:

- (a) Return all bottles to tray.
- (b) Place all waste material in the box.
- (c) Return the stencil to its place.
- (d) Wipe off the entire desk top with a **damp** sponge.
- (e) Place the stool under the desk.

8. **Microscopes.** Whenever possible each student is assigned an individual microscope. After assignment, the one using the microscope will be charged for any injury or neglect happening to the instrument during the regular scheduled laboratory periods at which he works. The microscope should be examined as soon as it is removed from the locker to see that it is in working order. The microscope must be returned to the locker at the end of the laboratory period.

9. Regulation Laboratory Periods. It is expected that the work assigned each day will be done in the regular laboratory period of two hours. The work is so arranged that this is possible for the average student. Students absent or having to do work outside of the regular laboratory hours for any valid reason, may arrange with the instructor to do such work at a time when there is not a regular class in the laboratory.

10. Laboratory Notes. All laboratory notes and drawings are to be made on the prescribed paper and in the following manner.

- (a) Each exercise number and title must be entered as in the manual.
- (b) All experiments are to be written up under the headings of
 - I. Object.
 - II. Method.
 - III. Results.
 - IV. Conclusions.
- (c) In presenting results, the data should be tabulated whenever possible.

The following conditions should be observed when recording results:

- 1. In writing up experiments use the indicative mode, passive voice, third person.
- 2. Do not use abbreviations.
- 3. Put down your own conclusions as well as answers to questions.
- 4. Include important details.
- 5. All exercises asked for must be in by Saturday noon of each week.
 - (d) Drawings should always be located on the left hand margin of the page. The outline stencil should be used for drawings of test-tube cultures and of microscopic preparations. All drawings should be properly labeled underneath and the description should be to the right of the same page.
 - (e) The pages of all the notes are to be numbered consecutively from the beginning of the course.
 - (f) Notes are to be placed on only one side of each sheet.
 - (g) The notes for each week's work should be placed in the regulation envelope when turned in. They will be graded and returned the succeeding week.

PART II.

STUDY AND CULTIVATION OF BACTERIA

Exercise 1. Construction and Operation of the Microscope

MATERIALS:

A compound microscope completely equipped

A test object mounted on a slide

1. The type of compound microscope used in bacteriological work represents the highest development in the fields of optics and mechanics. The mechanical parts are easily injured; the lenses, especially those giving great magnifications, are delicate and expensive. The instrument should be handled with care.

2. Notice that the compound microscope (Fig. 1) is provided with a *foot*, A; *arm*, B; *stage*, C; *tube*, D; *nose piece*, E; *objectives*, F and G; *Abbe condenser*, H; *iris diaphragm*, I; *ocular*, J; *mirror*, K; *coarse adjustment*, L; and *fine adjustment*, M.

FIG. 1—CONSTRUCTION OF THE COMPOUND MICROSCOPE

FIG. 2—EFFECT OF DIVERGENT RAYS ENTERING THE CONDENSER.

The rays come to a focus below the object and the illumination is reduced.

FIG. 3—EFFECT OF PARALLEL RAYS ENTERING THE CONDENSER.

The rays are brought to a focus in the plane where the object lies.

3. The Abbe condenser, H, receives the light falling on an area of the mirror equal in size to the lower lens of the condenser, and concentrates it on a much smaller area. (See Figs. 2 and 3). This increases the illumination and brilliance of the object, a result which is very important in the operation of high power objectives.

4. The *iris diaphragm*, I, provides a means by which the amount of light entering the front lens of the objective may be regulated. It is especially of value when using the low power objectives because with them the definition and detail of the object is brought out more clearly when the light is not too intense.

5. The *oil-immersion* objective gives the highest magnification. The front lens is very small and hence admits but little light. In order that the objects

FIG. 4—SHOWING THE PRINCIPLE OF THE IMMERSION OBJECTIVE

viewed with it may be seen distinctly, the light must be concentrated by means of the condenser, and be conserved by means of a liquid, e. g. oil of cedar, having the same index of refraction as glass. The oil fills the space between the object and the objective. In this way the loss of light is prevented as shown in Fig. 4.

6. The *ocular* or eyepiece, J, into which one looks when using the microscope, forms an image of the image formed by the objective; hence the term, compound microscope. Oculars of varying powers of magnification can be used, thereby enabling one to obtain any desired magnification, the practical working limit being 1200 to 1500 diameters.

7. The *mirror*, K, is used to reflect the light into the condenser. One side is plane or flat for use with daylight, and the other side is concave for use with artificial light. In either case parallel rays are reflected into the condenser. (See Figs. 2 and 3.)

8. Place the microscope in a *vertical* position on the desk with the coarse adjustment next you and near enough that you can look into the ocular easily.

9. Lower the tube with the coarse adjustment until the front lens of the objective is within 6 m. m. of the stage. See that the iris diaphragm below the condenser is open.

10. Remove the ocular. Swing the low power objective into position. While looking into the tube, adjust the mirror so that an image of the electric bulb is seen in the center of the lighted area, which is known as *the field*. Replace the ocular. The field should be uniformly bright. If it is not, alter the position of the mirror until it is uniformly lighted.

- (a) Turn the mirror slightly to the right. What is the effect?
Turn the mirror slightly forward. What is the effect?
- (b) Close the diaphragm. What is the effect?
- (c) Lower the condenser slowly while looking into the ocular.
What is the effect?

11. Whenever starting to use the microscope, examine it to determine that the iris diaphragm is open, the condenser is up as high as it will go and the concave side of the mirror is up. Place the instrument near the edge of the desk facing directly in. Adjust the light as described in paragraph 10. Do not move the microscope for the purpose of obtaining even illumination of the field. Use the mirror for this purpose.

12. Examine a permanent, mounted preparation of the leg and wing of the common house fly.

13. In **focusing** the microscope upon any object to be viewed, the proper objective and ocular being in place, the tube is moved down by the coarse adjustment, L, until the objective is near the object, but not touching it. Looking into the ocular, the motion is reversed. When the image begins to show, the movement must become slow.

14. With the lowest power a good focus can usually be obtained with the coarse adjustment. However, with the high powers the *fine adjustment*, M, must be used in order that the tube may be moved slowly. The fine adjustment works on a fine thread so that a complete revolution of the screw moves the tube very little. Care should be taken not to turn the screw completely down until it is "jammed" tight or so far upward that it is off the thread.

15. Place in the notes the following:

- (a) Make of the microscope.
- (b) The number of the ocular.
- (c) The numbers of the objectives.
- (d) The magnifications obtained with the ocular and each of the objectives on the microscope. See table below.
- (e) By what characteristic can one recognize at a glance the lowest power objective? the oil immersion objective?
- (f) Which way must the coarse adjustment be turned to raise the tube?
- (g) Which way must the fine adjustment be turned to raise the tube?
- (h) The condenser can be raised and lowered in two ways. What are they?
- (i) The image of the object viewed is an inverted one. What effect does this have on the direction the slide must be moved in order to move an object seen in the field from left to right?
- (j) The values given in the table of magnification refer to the apparent increase in length of a line as viewed under the microscope—linear magnification. From these values how would the magnification of an area be obtained?

16. The first five minutes of the next period will be devoted to a written quiz on the names applied to the various parts of the microscope and the purpose of each part.

TABLE OF MAGNIFICATIONS
(TUBE LENGTH OF 160 M. M.)

Objectives	Oculars			
	4x (2 in.)	5x (2 in.)	8x (1 in.)	10x (1 in.)
16 m. m. ($\frac{2}{3}$ in.)---	40	50	80	100
4 m. m. ($\frac{1}{8}$ in.)----	176	220	352	440
1.8 m. m. ($\frac{1}{12}$ in.)--	380	475	760	950

Exercise 2. Examination of Objects Under the Microscope

MATERIALS:

- 3 Plain glass slides
- 2 Cover glasses
- Clean sand
- Copper chloride crystals
- Cotton fibers
- Wool fibers

1. On a clean slide place near the center a *few* grains of sand and a *few* copper chloride crystals.
2. Adjust the microscope.
3. Place the slide on the stage of the microscope with the objects to be examined over the opening. See that the clamps hold the slide securely.
4. Swing the low power (16 m. m.) objective into position and lower the tube until the tip of the objective is within 4 m. m. of the object on the slide.
5. While looking into the microscope, slowly raise the tube by turning the coarse adjustment toward you until the objects come into view. It is well to move the slide a very little while focusing in order that the objects may more easily be detected. Do not mistake shadows of your eyelashes, or dust and lint particles on the lenses of the eyepiece for the objects to be examined. Turn the eyepiece to see if any such particles move. If so, they are on the eyepiece. If the lenses are very dirty, clean them with lens paper. If this does not remove the dirt, ask the instructor to clean them for you.
6. Try changing the amount of light by opening and closing the diaphragm. Draw the objects as seen with the 16 m.m. objective, remembering the regulations for drawings. (See page 2, paragraph 10.)
7. Place a large drop of water on a slide. In the water place a few wool fibers. Clean a cover glass. Hold it inclined so that the lower edge touches the slide and the edge of the drop. Lower it carefully in order that the space beneath the cover glass will be completely filled with water and not with a mixture of water and air. Remove excess of water with a blotter by blotting gently as when blotting ink. Examine the fibers with the 16 m.m. objective. Draw and describe them as seen with this objective.
8. Examine the fibers with the 4 m. m. objective. Draw them as seen with this objective.
9. Examine some cotton fibers in the same manner. Draw them as seen with the 4 m. m. objective.
10. Examine some threads, warp and woof, from your suit. Is it all wool?
11.
 - (a) How do the numbers of the objectives correspond to their distances from the objects when in focus?
 - (b) With what intensity of light can the details of the wool be most easily distinguished?
 - (c) What is the structure of the wool fiber as shown by its appearance under the microscope?
 - (d) What is the structure of the cotton fiber as shown by its appearance under the microscope?

Exercise 3. Examination of Stained Dead Bacteria

MATERIALS:

Prepared slides of the principal morphological types of stained, dead bacteria.

1. Adjust the microscope as before. See that the diaphragm is completely open and the lighting is even.
2. Place the slide of stained or dyed specimens of dead bacteria upon the stage of the microscope.
3. Examine preparation A with the 16 m. m. objective.
 - (a) Is there any detail or anything definite to be seen?
 - (b) Draw what can be seen.

FIG. 5—FORM AND ARRANGEMENT OF THE LOWER BACTERIA
A, spheres; B, rods; C, spirals

4. Examine the same preparation with the 4 m. m. objective.
 - (a) What can be seen?
 - (b) Draw a *few* of the objects seen.

Make the drawings large enough to show the exact shape of the objects and much larger in proportion to the size of the stencil than the objects bear to the microscopic field.

5. Raise the tube until the 4 m. m. objective is not less than 4 m. m. above the object. Place a small drop of immersion liquid on the center of the cover glass. Revolve the oil immersion objective into place. Carefully lower it, watching from one side, until the lens of the objective is immersed in the oil and almost touches the object.

6. While looking into the ocular, and while moving the slide slowly back and forth, turn the fine adjustment so that the tube moves upward. Turn slowly until the image appears sharp and clear.

7. Draw and describe preparation A as seen with the oil immersion objective. It is not necessary to draw all of the organisms seen in a field. The drawings should be large enough to show the exact shape of the organisms in outline.

8. Examine preparation B with the oil immersion objective. Draw a field from the preparation.

9. Examine preparation C with oil immersion objective. Draw a field from the preparation.

10. According to shape, name the bacteria in preparations A. B. and C. (See Fig. 5.)

11. Draw and describe the organisms in preparations D. showing spores and in E. showing flagella.

12. Knowing the diameter of the field of the oil immersion objective, (see paragraph 14), estimate the size in millimeters of the organisms in preparations A and B.

13. The micron (written μ) is the standard of measurement for bacteria. 1 micron—0.001 m. m.

(a) Give the estimated size of the organisms in microns. Show the calculations in each case.

14. The diameters of the real fields of the objectives most commonly used are given in the following table:

DIAMETER OF REAL FIELDS OF OBJECTIVES

Objectives.	Diameter of Field Using 10x (1 inch) Eyepiece and a tube length of 160 m. m.
16 m. m. ($\frac{2}{3}$ in.)	1.55 m. m.
4 m. m. ($\frac{1}{6}$ in.)	0.31 m. m.
1.8 m. m. ($\frac{1}{12}$ in.)	0.16 m. m.

Note.—As soon as the work with the oil-immersion objective is completed, it should be wiped off with lens paper.

Exercise 4. Microscopic Study of Living Bacteria

MATERIALS:

Carmine powder in water

Hay infusion

Whole milk

Pure cultures of bacteria in meat broth

Hanging-drop slide

Cover glasses

Vaseline

1. Clean a hanging drop slide. Place a ring of vaseline around the edge of the depression. Clean a cover glass and lay it on a clean piece of paper.

2. With the wire loop place a small drop, about the size of a pin head, of a suspension of *carmine* in water on the center of the cover glass.

Note.—Whenever the wire loop or needle is used, it must be sterilized *immediately* before and after using, in the manner shown by the instructor.

3. Invert the slide and carefully lower it until the cover glass is touching the ring of vaseline on all sides.

4. Quickly turn the slide right side up so that the drop is suspended (hangs)

FIG. 6—CORRECT HANGING DROP

FIG. 7—WRONG OR "FALLEN" HANGING DROP

over the depression in the slide. (See Fig. 6.) Carefully press down on the edge of the cover glass so that the vaseline makes a seal. Be careful not to cause the drop to fall or touch the bottom of the depression. (See Fig. 7.) In case this happens clean the slide and cover glass and repeat.

5. Examine the drop with the 16 m. m. objective. Shut off most of the light in order that the drop may be seen distinctly. Find the edge of the drop.

6. Raise the 16 m. m. objective a little and revolve the 4 m. m. objective into place. Regulate the light (usually by admitting more of it) until the field is grayish.

7. Looking at the objective sideways, carefully lower it until it nearly touches the cover glass.

8. Looking into the eyepiece, focus *upward* very slowly with the *fine adjustment* until the particles of dye are plainly visible. Try regulating the diaphragm until the best light is secured.

9. Examine the smaller carmine particles and groups of particles.

(a) What is their color and shape?

(b) Do they move?

(c) Is the movement vibratory, or do the particles move from one place to another in the preparation?

The vibratory form is called *pedesis* or *brownian* movement.

10. Examine some diluted milk (1 part whole milk to 2 parts water) in a like manner.

11. Examine a dark-field preparation of carmine particles. See the instructor's demonstration.

12. Great numbers of bacteria of different kinds will develop in a jar containing water and grass, hay or leaves, etc. The soluble substances in these diffuse into the water and serve as food for bacteria and other micro-organisms. The micro-organisms that were on the hay and leaves, in the water or adhering to the jar or those that are introduced with dust from the air develop with amazing rapidity. This development will take place at temperatures varying from 15 degrees C. to 40 degrees C. and is an example of what is taking place in nature wherever food and moisture permit the growth of bacteria.

Prepare a hanging drop from the infusion on the desk and locate the drop in the same way as before.

13. After locating the edge of the drop with the 16 m. m. objective, examine it with the 4 m. m. objective. Focus upward very slowly with the fine adjustment until the objects are in view. The organisms in the shoal water are the most easily studied. The large, round, oval and oblong objects that seem to move about so rapidly are not bacteria, but animals. Animals like these are known as protozoa. The small, round, rod, or spiral-shaped individuals present are the bacteria.

14. Draw and describe a field as seen in the hanging-drop preparation.

- (a) Are there a number of kinds of bacteria in the infusion? Give reasons for your answer.
- (b) Can any of them move? Indicate the path of the movement of individual organisms by dotted lines and arrows.
- (c) What seems to be the nature of their movement? Notice the apparent vibration of some of them that are not moving from place to place.

15. Examine in hanging drop each of the pure cultures of bacteria furnished; draw and describe each.

16. From the observations of the cultures, classify each one according to shape. (See Fig. 5).

- (a) Unstained, living bacteria have been examined as well as stained, dead bacteria. Which one of these preparations is the easier to study? Why?
- (b) Can flagella be seen on the bacteria in any of the preparations Give the reason for your answer.
- (c) Can dead bacteria be detected by observation of a hanging drop? Give reasons for your answer.

Exercise 5. Microscopic Study of Living Yeast and Molds

MATERIALS:

Culture of yeast on dextrose agar

Cultures of molds on potato agar

1. Place three or four drops of the yeast suspension upon a plain slide. Cover with a clean cover glass. The cover glass should float on the liquid which should be sufficient in amount to extend under the cover glass, but should not cover the upper surface so that it comes in contact with the objective.

2. Examine the preparation with a 4 m. m. objective. Draw and describe the organisms.

3. Examine the pure cultures of molds in the Petri dishes. Describe their naked eye appearance.

4. Place the dish on the stage of the microscope with the cover removed. Examine the patches, preferably the edge, with the 16 m. m. objective. Notice the color, form, and size of the patch of mold. Notice the filaments which grow on the jelly-like food substance and the erect ones carrying the spores.

5. Make a more detailed examination of the filaments and spores using the 4 m. m. objective.

6. Make a large detailed drawing of each of the molds furnished. In each case show the following:

- (a) Mycelium and filament structure.
- (b) Fertile hyphae.
- (c) Sporangia and spores.
- (d) Label all of the parts and give the genus of each mold studied.

7.
 - (a) How do molds differ from bacteria.
 - (b) How do they differ from yeasts?
 - (c) How do the molds reproduce?
 - (d) How yeasts reproduce?
 - (e) How do yeasts compare in size with bacteria?

Exercise 6. Making Stained Preparations of Bacteria

MATERIALS:

Hay infusion

Cultures of bacteria on agar

Clean slides

Clean cover glasses

Canada balsam

Tooth picks

1. Examine the infusion with the naked eye. Note whether it has changed since it was used in Exercise 4. Look up the meaning of zoogloecae in Appendix B.

2. Wet the tip of one finger, rub it on a cake of Bon Ami, and with the same finger spread the paste thus formed over the surface of the slide. Allow the paste to dry, and wipe it off with a *clean* dry cloth or paper towel. If the slide is clean, a drop of water placed on it will spread in an even film over the glass. Grease causes water to collect in tiny drops.

Place a small drop of the infusion in the center of the clean slide. Spread the drop over an area about the size of a dime. (See Fig. 8.) The wire loop can best be used for this purpose.

FIG. 8—MAKING A SPREAD ON A SLIDE

3. Permit the spread on the slide to dry. Do not heat the slide to hasten the drying.

4. Pass the slide, film side up, quickly three times through the Bunsen flame. (See Fig. 9.) The heat kills the organisms and causes them to adhere to the glass throughout subsequent operations. It is termed “fixing” the preparation.

FIG. 9—FIXING THE ORGANISMS TO THE SLIDE

5. Lay the slide on the sink cover for staining and pour enough methylene blue solution on it to cover the spread as is shown in Fig. 10.

Permit the stain to act three minutes, and then wash the preparation with tap water.

FIG. 10—STAINING BACTERIA

6. Dry the preparation between blotters.

7. *Label the slide*, being careful to place the label at one end on the same side as the spread.

Note.—This kind of a preparation answers very well for all temporary work where preparations are not desired for permanent use.

In order to make the preparations more nearly permanent, the smear or spread may be covered with a very thin piece of glass, a cover glass, which may be cemented to the slide with Canada balsam. The preparation should be dry before the Canada balsam is placed on it. If immersion oil has been used on the preparation, it should be removed by flooding the spread

twice with xylol, this being allowed to act a few moments each time before it is drained off, after which treatment, the slide is allowed to dry. The Canada balsam, dissolved in xylol, should be of such a consistency that a drop one-eighth of an inch in diameter will spread under the weight of the cover glass so as to fill the space beneath the cover glass completely. The cover glass should not be dropped on the Canada balsam, but should be held in an inclined position and very slowly lowered on the drop. The preparation should be placed in a horizontal position for several days until the balsam has hardened.

8. Remove some of the tartar from the teeth with a tooth pick and spread in a thin, even film without water. Fix and stain three minutes with methylene blue.

9. Prepare a spread of some old milk which is not curdled. Be sure the spread is thin. Do not dilute with water. Dry and immerse in xylol three to five minutes to remove the fat. Wash in alcohol to remove the xylol. Wash in water. Stain three to five minutes with methylene blue.

10. From each of the pure cultures of bacteria furnished make a preparation in the following manner:

With the wire loop place a small drop of tap water on a clean slide. Transfer a minute portion of the bacterial growth on the solid nutrient agar to the drop of water by touching the growth with the tip of the wire needle. Mix the bacteria with the water, which should then be spread in an even film over an area as large as a dime. The turbidity produced in the drop of water by mixing the bacteria in it should be evident to the eye. Allow the preparation to dry. Fix it and stain with the dye indicated by the instructor.

11. Examine the preparations in order, as was done in Exercise 3, using the oil immersion (1.8 m. m.) objective only. *Draw and describe* one good field from each preparation.

- (a) What can you see in the infusion?
- (b) How can you tell bacteria from debris?
- (c) Can you tell the protozoa from the bacteria?
- (d) How many different forms of bacteria are visible? Compare them with Fig. 5.
- (e) What can be seen in the tartar?
- (f) Do spheres, rods or spirals predominate?
- (g) Are there many different forms of bacteria in milk?
- (h) What is the amorphous blue material in the stained milk spread?
- (i) What morphological types of bacteria were found in the pure cultures?
- (j) What is the value of anilin dyes in the study of bacteria?

Exercise 7. Sterilization

MATERIALS:

- Beef broth
- Skimmed milk
- Eight empty test tubes
- Two sterile test tubes
- Cotton batting

Note.—Where the test tubes contain cultures and experimental material, they are handled in tumblers, and to avoid breaking the tubes a cushion of cotton is placed in the bottom of each tumbler.

1. Prepare five tubes of milk and five tubes of beef broth according to the following outline, making plugs as shown by the instructor.

No.	Material	Kind of tube	Treatment
1a 1b	beef broth milk	sterile tube plugged	not heated
2a 2b	beef broth milk	unsterilized tubes	not plugged not heated
3a 3b	beef broth milk	unsterilized tubes, plugged	heat to 100° C. for ten minutes
4a 4b	beef broth milk	unsterilized tubes, plugged	heated to 120° C. for 30 minutes
5a 5b	beef broth milk	unsterilized tubes, plugged	heat to 120° C. and inoculate

2. Treat tubes 3a and 3b in a cup of boiling water 10 minutes over the Bunsen flame.

3. Prepare a labeled piece of paper giving the experiment and desk number. Fasten this paper around the tubes 4a and 4b, and 5a and 5b with a rubber band. Place them in the autoclav to be heated for 30 minutes at 116°-120°C.

FIG 11-CORRECT METHOD OF HOLDING TUBES WHEN TRANSFERRING CULTURES
Note how the plugs are held between the fingers of the right hand. The plugs are *never* laid down. The tubes are held as nearly horizontal as possible.

4. When cool, tubes 5a and 5b should be inoculated with a loopful of soil or tap water. (See Fig. 11.)
5. Examine the tubes at each opportunity for two weeks. Tabulate the data as shown in Table I on the regular note paper.
6. At the end of the observation period stain some of the milk and broth from tubes 4a and 4b, and 5a and 5b. Use carbol fuchsin, one minute for the broth, and methylene blue three minutes for the milk.

TABLE I.—Sterilization of Milk and Broth

Tube No.	Treatment of beef broth	Appearance after two days	Appearance after one week	Appearance after two weeks
1a 2a				
3a 4a				
5a				

Tube No.	Treatment of milk	Appearance after two days	Appearance after one week	Appearance after two weeks
1b 2b				
3b 4b				
5b				

7. Describe efficient methods for the sterilization of milk and broth.
- (a) What was the macroscopic evidence that any milk or broth tubes were not sterile? Microscopic?
 - (b) What does “sterile” mean? Which tubes were sterile?
 - (c) Why is an autoclav temperature of 116° C more efficient than hot air oven temperature of 150° C?
 - (d) Give a satisfactory method of sterilizing water, milk, Petri dishes, bandages, a room.

Exercise 8. The Distribution of Bacteria

MATERIALS:

A case of sterile pipettes

One sterile, empty test tube

Five sterile Petri dishes

Five tubes of sterile, meat extract-peptone agar

Soil suspension

1. Label the five dishes near the edge of the covers. Be very careful not to handle the dishes any more than is necessary and remove the covers only when necessary.

2. Mark the labels as follows:

(a) "Air"

(b) "Finger tips"

(c) "Sterile"

(d) "Soil suspension, one drop"

(e) "Tap water, one cubic centimeter"

3. With a sterile pipette place a drop of the soil suspension in dish (d).

4. Collect some tap water in a sterile test tube.

5. With another pipette place one cc. of tap water in dish (e).

6. Melt five tubes of agar (bacterial food in a jelly) in the metal cup by boiling them in water. When the agar is melted, cool to 45°C . by mixing cold water carefully with the hot water in the cup. Great care must be exercised not to cool the water below 45°C . At this temperature bacteria can be mixed with culture media without injuring them.

FIG. 12—THE CORRECT METHOD OF POURING LIQUEFIED CULTURE MEDIA INTO PETRI DISHES

After wiping the water from the outside of a tube of agar, remove its plug, flame the mouth of the tube in the gas flame and pour the agar into dish (a), (See Fig. 12). Tip the dish carefully from side to side to spread the agar over the entire bottom of the dish. Place the dish on a level part of the desk until the agar becomes solidified. In the same way pour agar into the other dishes.

7. Expose dish (a) with the cover off to the air of the laboratory for twenty or thirty minutes. Leave the cover right side up by the side of the dish during the exposure.

8. Carefully touch the finger tips and nails of one hand to the surface of the agar in dish (b). Keep dish (c) closed.

9. When the agar is solid and dish (a) is exposed, invert the plates in the 28°C incubator for one week.

10. Rinse out pipettes used and leave on the desk to be sterilized. Wash tubes that contained agar, and invert in the desk.

11. Examine the plate cultures. The differently colored and shaped spots are colonies of bacteria. Where one colony is separate from the others, uniform in shape, color, and appearance, it is probably a colony or group of organisms that has grown from a single cell.

Select three colonies with the instructor's approval and mark them 1, 2, 3 respectively with a pen or with a wax pencil.

12. Examine them with the 16 m.m. objective. Place the Petri dish, inverted, cover and all, on the stage of the microscope. Draw and describe them according to the outline in Appendix A.

- (a) What is the difference in appearance of the agar in the dishes,
(1) as compared to the time when poured into the dishes;
(2) as compared to each other after one week?
- (b) How may the appearance of the dish labeled "sterile" be explained?
- (c) What is the advantage of plate cultures?
- (d) Who first introduced the plate culture method?
- (e) Why do colonies not form in liquid media?
- (f) Is it always true that each colony forms from one cell? Give reasons for your answer.

Exercise 9 Isolation of Pure Cultures of Bacteria

MATERIALS:

- 3 tubes of plain agar for slopes
- 3 tubes of plain broth

1. Pure cultures are to be made from the colonies described in Exercise 8. A pure culture of a bacterium, yeast or mold is a culture which contains only **one kind** of a bacterium, yeast or mold. It is assumed that each of the three colonies selected have originated from a single organism.

2. Mark three tubes of agar, 1, 2, 3 respectively. If the agar is not sloped in the tubes, melt and slope it as shown by the instructor.

FIG. 13—"FISHING" OFF COLONIES FROM A PETRI DISH CULTURE

3. When the agar has become firm, inoculate tube No. 1 from colony No. 1 (Exercise 8) as follows: Heat the straight wire needle in the flame to redness to sterilize it. When cool, touch the tip of the needle to the colony

FIG. 14—MAKING A STREAK CULTURE FROM A COLONY

(Figs. 13 and 14) and spread the adherent organisms on the agar in the tube by drawing the needle *once* lightly over the surface of the agar from the bottom to the top. Be careful that the needle does not touch any other colony during the operation. Inoculate the other two tubes of agar from the respective colonies.

4. Inoculate the broth tubes from the same colonies, rinsing the organisms from the needle in the broth.

5. Leave the cultures in the incubator for 48 hours or in the locker for four or five days.

6. Examine the cultures. Look for a streak along the needle track on the agar slopes, and for turbidity, sediment, or a surface film in the broth cultures. Draw and describe the cultures according to the outline for broth and agar streak cultures in Appendix A.

7. Examine the organisms in each broth culture by means of the hanging drop and determine if they are motile. Record the morphology of the organisms in each culture.

8. (a) What are some other kinds of agar tube cultures besides slant cultures?

(b) What are the advantages of solid media for tube cultures as compared to liquid media.

Exercise 10. The Microscopic Examination of Bacteria in Pure Culture

MATERIALS:

3 plain agar slants

Pure cultures made in Exercise 9

9 plain slides

1. Carefully label three plain agar slants and make new transfers of the pure cultures isolated in Exercise 8. Place them in the incubator for 48 hours. Save them for use in Ex. 11.

2. Carefully clean 8 slides. Arrange three of them in a row and label each of the three the same as one of the pure cultures that have been isolated. Place a small drop of water, about 1/8 inch across, in the center of each slide. Remove some of the growth from the agar culture with the sterile wire needle and mix it with the water on each slide, spreading the mixture as thin as possible. (See Fig. 8.) Permit each to air dry. Cause the bacteria to adhere to the slides by passing them three times through the top of the gas flame (fixing). (See Fig. 9.) Lay the slide upon the wire cover of the sink for staining. (See Fig. 10.) Repeat with each of the other cultures.

3. Stain one slide from each culture with methylene blue for 5 minutes. Stain another slide from each culture with carbol fuchsin for one minute. Stain the remaining slide from each culture by means of the Gram method as follows:

- (a) Stain with ammonium oxalate crystal violet for 2 or 3 minutes.
- (b) Pour off the stain.
- (c) Do not wash.
- (d) Treat with Gram's iodine (Lugol's) solution for one minute. Pour off and treat again for one minute.
- (e) Pour off iodine. Wash in water *quickly*. Blot off excess water quickly but do not permit the film to air dry.
- (f) Decolorize in equal parts of acetone and 95 per cent ethyl alcohol, until the stain no longer washes from the slide.
- (g) Dry between blotters.
- (h) Counterstain one minute with aqueous one per cent safranin.
- (i) Wash and dry between blotters or air dry.

This method of staining is an aid in the classification of bacteria. Some bacteria do not lose the purple color imparted to them by the crystal violet when treated by this method; others do lose the color. The former are termed Gram-positive, the latter Gram-negative organisms.

4. Examine the slides. Draw and describe one slide from each culture.

- (a) What is the form of the organisms from each culture? (Fig. 5.)
- (b) Are any of them yeasts?
- (c) Do all of the organisms stain by the Gram method?
- (d) Are there spores in any of the cultures?
- (e) What stain shows each organism best?
- (f) Under the microscope, how can bacteria be distinguished from yeast?
- (g) In a Petri dish culture there is a mixture of bacteria, yeast, and mold colonies. What should be done and what would be found in distinguishing them?

Exercise 11. Enzyme Production by Microorganisms

MATERIALS:

Garden soil suspension
5 tubes of plain agar
5 tubes of starch agar
5 tubes of sterile milk
4 tubes of sterile gelatin
Pure cultures of bacteria
Sterile pipettes
10 sterile Petri dishes

A. Carbohydrate Hydrolyzing Enzymes (*carbohydrases*) Amylase.

1. Melt the starch agar and cool to 45° C.
2. Label five sterile Petri dishes as follows:
 - (a) Starch agar, soil suspension.
 - (b) Starch agar, pure culture 1.
 - (c) Starch agar, pure culture 2.
 - (d) Starch agar, pure culture 3.
 - (e) Starch agar, *B. graveolens* culture.
3. By means of a sterile pipette place a drop of soil suspension in Petri dish (a).
4. Immediately pour starch agar into all of the Petri dishes being careful to observe the precautions indicated in Exercise 8.
5. When the agar is solid inoculate the four remaining dishes with the organisms indicated. Use the straight needle and make the inoculation at one spot in the center of the dish.
6. Incubate the cultures inverted at room temperature for 48 hours.

B. Protein Hydrolyzing Enzymes (*proteinas*) Caseinase

1. Label five Petri dishes as follows:
 - (a) Milk agar, soil suspension.
 - (b) Milk agar, pure culture 1.
 - (c) Milk agar, pure culture 2.
 - (d) Milk agar, pure culture 3.
 - (e) Milk agar, *B. graveolens* culture.
2. With a sterile pipette, place one cc. of sterile milk in each of the Petri dishes.
3. By means of a sterile pipette place a drop of soil suspension in Petri dish (a).
4. Melt five tubes of plain agar and cool to 45° C.
5. Immediately pour agar into all of the Petri dishes being careful to mix agar and milk until the whole has a uniform white appearance.
6. When the agar is solid, inoculate the agar at one point in the center of each dish. Use the straight transfer needle, and make inoculations as designated in paragraph A5.
7. Incubate the cultures inverted at room temperature for 48 hours.

Gelatinase

1. Prepare stab cultures in gelatin using the pure cultures 1, 2, and 3 from Exercise 10 and the culture of *B. graveolens*.
2. Be sure the gelatin is solid. If it has the slightest tendency to become soft, the tubes should be placed in a glass of ice-water until solid.
3. Make the stab culture in the gelatin by thrusting the inoculated straight wire into the center of the solid medium, nearly to the bottom of the test tube. Be sure the wire is perfectly straight.
4. Incubate the cultures in the 20° C. incubator to prevent melting of the gelatin. Examine after one week.

Observation of Cultures

1. Flood the starch agar plates evenly with Lugol's solution and drain dry.
2. Examine both the starch agar and milk agar plates for halos around the colonies. Notice the clear, yellow or wine red color of the halos in starch agar.
3. Examine, draw and describe the gelatin stab cultures. See Appendix A, Part B, pages 77 to 80.
 - (a) What causes the clear halos around the colonies?
 - (b) How do you account for the enzyme action so far from the colonies in some instances?
 - (c) What is the value of these enzymes to the bacteria?
 - (d) How are these bacterial enzymes of value to man?
 - (e) Were any molds observed to produce enzymes?
 - (f) What are the common names applied to the enzyme changing starch to maltose?
 - (g) What kind of chemical change is produced by the enzymes studied in the exercise?
 - (h) Name three other types of changes caused by enzymes?
 - (i) State your conception of an enzyme?
 - (j) Why is agar used for streak cultures more than gelatin?
 - (k) Why is gelatin preferred to agar for stab cultures?

Exercise 12. The Fermentation of Carbohydrates by Bacteria

MATERIALS:

2 fermentation tubes of dextrose broth containing brom cresol purple

2 Durham tubes of lactose broth containing brom cresol purple

1 culture each of *Streptococcus lactis* and *Aerobacter aerogenes*

1. Inoculate one Durham tube of lactose and a fermentation tube of dextrose broth with each of the organisms furnished.

2. Place the cultures in the incubator and examine them in 48 hours.

3. Observe the reaction of the broth in the tubes. Note whether gas has been formed in both lactose and dextrose broth. If gas has been formed in the fermentation tubes, measure it and determine its composition as shown by the instructor.

4. Compare the color of the indicator in the Durham tubes with a set of standard solutions containing brom cresol purple. Record the change in reaction as shown on the pH scale of hydrogen ion concentrations. (See Fig. 15.)

- (a) What is the range in pH of brom cresol purple?
- (b) What was the change in the reaction of the sugar broths where the bacteria grew? (See the diagram Fig. 15).
- (c) What acids do bacteria most commonly form from dextrose and lactose?
- (d) What was the gas formed in the fermentation tube?
- (e) What gaseous substances are usually formed in the decomposition of carbohydrates?
- (f) What are the steps in the conversion of an insoluble carbohydrate like starch into acid by bacteria?
- (g) Why is the bacterial decomposition of carbohydrates of more industrial importance than that caused by molds?
- (h) What is the advantage to bacteria of being able to ferment carbohydrates?
- (i) How does the action of yeast on sugars differ from the action of bacteria?

FIG. 15—DIAGRAMMATIC REPRESENTATION OF THE pH SCALE OF TRUE ACIDITY

The pH values, given as the log of $\frac{1}{[\text{H}^+]}$, are shown on the numbered horizontal lines. The acid end is at the top, the alkaline at the bottom. The diagonal line shows the proportion of H ions to OH ions at each pH. Thus neutrality where $\frac{\text{H}}{\text{HO}} = 1$ is at pH 7.0. The true acidity given in pH values for some familiar substances is indicated. The working range of brom-cresol purple is shown by the curved line.

Exercise 13. The Production of Pigment by Bacteria

MATERIALS:

6 plain agar slants

6 glucose agar slants

3 plain gelatin for stab cultures

3 glucose gelatin for stab cultures

One culture each of *Ps. prodigiosus*, *Ps. flurorescens* and *Aerobacter aerogenes*.

1. Prepare one plain gelatin and one glucose gelatin stab culture from each of the cultures as follows: Make sure the transfer needle is straight. Sterilize the needle, cool, and touch the tip to the bacterial growth. Carefully make a stab with the inoculated needle in the center of the gelatin. The stab should be made parallel to the sides of the tube, and within a few millimeters of the bottom.

2. Place the three cultures, labeled and held together by means of a rubber band, in ice-water until they can be placed in the 20°C incubator.

3. Prepare two plain and two glucose agar slant cultures of each of the organisms.

4. Place one plain agar slant and one glucose agar slant of each culture at room temperature. Incubate the other plain and glucose agar slants at 37°C.

5. Examine the cultures every 48 hours for one week.

6. At the end of a week record, in table form, the absence of pigment as — and different degrees of pigment as +, ++, or +++. *Brightness of pigment should be considered and not the amount of growth.*

7. Place a few cc. of each of distilled water, chloroform, and carbon bisulphide in small test tubes. Add a large loopfull of the brightest pigmented culture to each. Observe any solubility in the solution.

8. (a) Where in the cultures is the pigment seen most conspicuously?

(b) How may this be explained?

(c) What composition of the medium is the most favorable for pigment production?

(d) What temperature is most favorable for pigment production?

(e) What does this suggest about the habitats of the organisms?

(f) What is the relation of oxygen to pigment production?

(g) What cultures showed this?

(h) What pigments are water soluble?

(i) What are pigment-producing organisms called?

(j) What is the practical agricultural significance of pigment-producing organisms?

Exercise 14. The Influence of the Composition of the Substance on the Types of Fermentation that will Occur in It.

MATERIALS:

- 4 100 cc. portions of sterile broth
- 8 sterile test tubes
- 1 300 cc. flask of sterile sand
- 4 sterile Petri dishes
- N/10 sulphuric acid
- Sterile glucose solution
- Pure cultures of *Psuedomonas fluorescens*, *Saccharomyces cerevisiae* and *Aspergillus* species.

1. Treat four 100 cc. portions of sterile broth, pH 7.0 as follows:
 - No. 1. Leave as it is.
 - No. 2. Add 5 cc. of 20 per cent glucose solution.
 - No. 3. Add 1 cc of N/10 sulphuric acid, giving a reaction of approximately pH 4.5.
 - No. 4. Add glucose and acid.
2. Mix well with gentle shaking; and carefully pour two sterile test tubes, one-third full from each flask. Be sure the tubes are labeled properly.
3. Pour each of four sterile Petri dishes one-third full of sterile sand.
4. Carefully label the dishes and moisten the sterile sand to saturation with one of the broths prepared above.
5. Inoculate the tubes and dishes as follows:

Broth No.	Tubes		Dishes
	1	2	
1	Bacteria	Yeast	Mold
2	Bacteria	Yeast	Mold
3	Bacteria	Yeast	Mold
4	Bacteria	Yeast	Mold

Use a culture of *Psuedomonas fluorescens* for the bacteria, *Saccharomyces cerevisiae* for the yeast and *Aspergillus* for the mold.

6. Incubate the cultures in the desk for two weeks. Examine them at the end of the first and second weeks for signs of growth. Construct a table showing the type of fermentation that has taken place in each case. Record the presence of gas, odor and the reaction.
7. (a) What do the results of this exercise indicate in regard to the conditions favoring and opposing the growth of bacteria, yeasts, and molds?
(b) Construct a diagram of the pH range of hydrogen ion concentration showing the optima for bacteria, yeasts, and molds? (See Fig. 15).

(c) The composition of milk, broth, grape-juice, and lemon juice is as follows:

MILK	
Water	87.10 per cent
Casein and Albumen	3.40 " "
Fat	3.90 " "
Milk Sugar (lactose)	4.75 " "
Ash constituents	0.75 " "
Reaction pH	6.8

MEAT BROTH	
Water	98.70 " "
Peptone	1.00 " "
Meat Extract	0.30 " "
Reaction pH	7.0

GRAPE JUICE	
Water	82.90 " "
Sugar (dextrose)	15.00 " "
Tartaric Acid	1.00 " "
Soluble Nitrogen	0.40 " "
Ash	0.70 " "
Reaction pH	4.50

LEMON JUICE	
Water	94.30 " "
Sugar	0.20 " "
Citric Acid	5.00 " "
Ash	0.50 " "
Reaction pH	2.20

What determines the changes and the kinds of organisms causing the changes in the above materials?

- (d) Why does milk undergo an acid fermentation?
- (e) What is the source of the acid in the lemon and grape juice?
- (f) What kind of change took place in the broth? Why?
- (g) What characteristics of natural organic substances determine the possibility of their decomposition by microorganisms?
- (h) What relation do these facts bear to agriculture?

PART III

SOIL BACTERIOLOGY

Exercise 15. The Number and Kinds of Bacteria in Different Soils

MATERIALS:

9 tubes sodium caseinate agar

9 sterile Petri dishes

Case sterile pipettes

3 9 cc. sterile water blanks

1 99 cc. sterile water blank

Suspension of soils, 1 gram of soil in 1000 cc. of sterile water

There are enormous numbers of bacteria in most fertile soils, and on this account a very small amount of the soil must be used in determining the number of bacteria. Results of determinations are always given per gram of soil, usually calculated on a "dry" basis. In all of the following work two representative soils will be used. One sandy loam, "soil A," and one black garden soil, "soil B." Prepare cultures from one soil.

1. Observe that there are 1 to 1000 dilutions of the two soils on the desk.

2. Prepare 4 quantitative dilutions of one soil and make duplicate plate cultures with 1-1000, 1-10,000, 1-100,000, and 1-1,000,000 gram of soil. Prepare a "blank" without the soil. Label dishes with the name of the soil and the dilution used.

FIG. 16—MANNER OF MAKING THE SOIL DILUTIONS

3. Proceed by arranging the water blanks and Petri dishes as shown in Fig. 16. Make all transfers with sterile 1 cc. pipettes as indicated by the continuous arrows, and transfer 1 cc. each time. All steps marked "A" are to be carried out with the first pipette and in the order given; steps marked "B" with the second pipette, etc.

4. Shake the 1 to 1,000 gram dilution well with a rotary motion, at least fifteen times. While the liquid is still in motion, transfer 1 cc. to the necessary

water blank or Petri dish. Be sure each dish receives the dilution for which it is labeled. Prepare the blank as shown.

- 5. Melt 9 tubes of plain agar and cool them to 45° C.
- 6. Pour the plates, being very careful to get the dilution water well mixed with the agar. Place the poured plates on a level surface while the agar hardens. Rinse out the tubes with hot water while the agar remaining in them is still soft.
- 7. When the agar is solid, incubate the dishes at room temperature. Examine the plates in 4 to 6 days.
- 8. Assuming that each colony has grown from a single organism, count the colonies on the best plates and estimate the number of organisms per gram of soil.

Plates containing between 70 and 200 colonies give the most accurate results and incidentally are the most easily counted.

- 9. Record the results on the regular note paper in tabular form like the following:

TABLE II.—Bacterial Content of Soil

Dilution	Number of Colonies on Plate	Number of Bacteria Per Gram Average of two plates	Apparent Number of Kinds from Gross Appearance	Number of Molds
1/1000 g.				
1/10,000 g.				
1/100,000 g.				
1/1,000,000 g.				
Blank				

- (a) From the results, can you say that there are many bacteria in average soils?
- (b) Are there many kinds present? Did all of the organisms present develop? Why? Give two good reasons for the answer.

- (c) Were there any organisms developing in the dish marked "blank"? What does this show?
- (d) How do the number and kinds of organisms in the soil examined compare with the results of the examination of the other soil?

Exercise 16. Ammonification by Soil Bacteria

MATERIALS:

- 3 tubes of beef broth
- 3 tubes of casein solution
- 3 tubes of urea solution
- Soil samples

The protein of plant and animal tissues and urea ($\text{CO}(\text{NH}_2)_2$), in which compound a great deal of the nitrogen in urine is found, readily undergo ammonification. Since carbon dioxide (CO_2) is practically always present in decomposing materials, the ammonia formed unites with it to form ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$).

1. Label each of the 3 tubes of beef broth, casein and urea medium with the name of medium, and the soil with which it is to be inoculated.
2. Inoculate *one* of each three tubes with a small quantity of one of the soils used in exercise 15, by moistening the wire loop with tap water and thrusting it into the soil. The amount of soil that will adhere to the moist loop is sufficient. Another tube of each three is to be inoculated with the other soil. The remaining tube is left without inoculation and is to serve as a control.
3. After 48 hours incubation at room temperature, test the *inoculated* tubes for ammonia by placing a drop of Nessler solution in a depression on a spot plate and adding to it one loopful of the culture. The presence of ammonia is indicated by the development of a color, a faint yellow with small amounts of ammonia, a deep yellow or brown with larger quantities and a brownish precipitate with still larger quantities.
4. After incubation for one week test both the inoculated and control tubes for ammonia.
5. Record the results of both tests on the regular note paper as in Table III. Rank the solutions according to the amount of ammonia present, the solution containing the smallest amount being ranked 1.

TABLE III.—Formation of Ammonia by Soil Bacteria

	Rank after 48 hours		Rank after 1 week		Control
	soil A	soil B	soil A	soil B	
Beef Broth					
Urea					
Casein					

6. In recording the conclusions, answer the following questions:
 - (a) From the above data, what is to be concluded as to the cause of ammonia formation in soils? Why?
 - (b) What was in the casein solution and beef broth that yielded ammonia upon decomposition?
 - (c) Give the most common sources of such substances in farm practice.

- (d) Do the ammonifying organisms render nitrogen available to plants as food? Explain.
- (e) From which one of the substances would nitrogen be made most quickly available to the green plant? Why?
- (f) In which of the tubes is ammonia made evident by odor?

Exercise 17. Nitrification

MATERIALS:

2 100 cc. Erlenmeyer flasks
 4 one-fourth pint milk bottles
 Ammonia and nitrite media
 25 cc. graduate
 Soil samples
 Milk bottle caps
 Cotton

Nitrification includes the oxidization of ammonia (NH_3) to nitrites (N_2O_3) and their subsequent oxidization to nitrates (N_2O_5). The organisms which carry out the oxidization of ammonia to nitrites and nitrites to nitrates are present in most cultivated soils. Their activity in a given soil may vary with the cultivation and cropping of the soil.

The solutions used for the cultivation of these organisms contain no organic matter—only in organic salts.

A. Nitrite Formation

In the culture medium for the nitrite-forming organisms, the source of nitrogen is ammonium sulphate (NH_4)₂SO₄ and in the beginning the solution contains no **nitrites** or **nitrates**.

Ammonium sulphate (NH_4) ₂ SO ₄)	2.00 grams
Di-basic potassium phosphate (K_2HPO_4) --	1.00 “
Magnesium sulphate (MgSO_4) -----	0.50 “
Ferrous sulphate (FeSO_4) -----	trace
Sodium chloride (NaCl)-----	0.40 “
Water -----	1000.00 “
Magnesium carbonate (MgCO_3)-----	excess

1. See that 3 milk bottles are clean
2. The culture solution is on the desk in the white enamel graduate and is labeled “ammonia medium.”

Stir thoroughly until the undissolved substances are in uniform suspension. Transfer by means of the 25 cc. graduated cylinder enough (about 10cc.) of the culture solution to each milk bottle and to the flask to form a layer of about one-fourth inch deep.

3. Inoculate one bottle with 2 or 3 measurefuls of soil A and another with 2 or 3 measurefuls of soil B. Inoculate the solution in the flask with both soils. Label the flask in pencil on the etched portion. Be sure the label states the desk number as well as the notations about the experiment.

4. Leave the flask properly plugged and labeled on the desk to be sterilized.
5. Test each culture and the control once a week for:

- (a) Ammonia
- (b) Nitrites

When any culture no longer gives a positive test for nitrites, test it for nitrates.

B. Nitrate Formation

In the culture medium for the nitrate-forming organisms, the source of nitrogen is nitrite and the solution contains *no nitrates* at the beginning.

Sodium nitrite (NaNO_2)	1.00 gram
Di-basic potassium phosphate (K_2HPO_4)	1.00 gram
Magnesium sulphate (MgSO_4)	0.30 gram
Sodium carbonate (anhydrous) (Na_2CO_3)	1.00 gram
Sodium chloride (NaCl)	0.50 gram
Ferrous sulphate (FeSO_4)	trace
Water	1000

1. Place in each of two clean bottles and a flask enough of the solution marked *nitrite medium* to form a layer one-fourth inch deep. Inoculate the bottles and flask as described in paragraph 3 above.

2. Incubate all the cultures in the desk.

3. Test the cultures once each week for:

(a) Nitrites.

(b) When Trommsdorf's solution no longer gives a test for nitrites, test for nitrates.

Note—To test for ammonia.—Place in a depression on a spot plate one drop of Nessler's solution. Touch with a sterile glass rod carrying a drop of the solution to be tested. Do not stir. A test gives a brown color.

To test for nitrites.—Place in a depression on a spot plate, Trommsdorf's solution one or two drops and 1:3 sulphuric acid two or three drops. Mix. Touch with a drop of the solution to be tested. Do not mix.

A test gives a deep blue color.

To test for nitrates.—Place in a depression on a spot plate one drop of a solution of diphenylamine in concentrated sulphuric acid. Touch with a drop of the solution to be tested.

A test gives a very dark blue color. Nitrites also give a blue color with diphenylamine, therefore before one can be certain nitrates are present, the solution must be tested for nitrites by the Trommsdorf reagent and the absence of nitrites shown.

4.
 - (a) What do you conclude regarding the nitrifying power of the two soils studied?
 - (b) What agricultural practice tends to promote the activity of these organisms in the soil? Why?
 - (c) What is the purpose of the magnesium carbonate in the solution containing ammonium sulphate?
 - (d) What use does the nitrite-forming organism make of the ammonium sulphate?
 - (e) What use does the nitrate-forming organisms make of the sodium carbonate?
 - (f) Why is plenty of air necessary for the growth of these organisms?
 - (g) Who first isolated pure cultures of these organisms?
 - (h) What are the names of the two species of organisms concerned in these processes?
 - (i) Why is it not necessary to add magnesium carbonate to the solution containing sodium nitrite?

TABLE IV.—Nitrification by Soil Bacteria

Time in Weeks	Ammonia Medium									Nitrite Medium					
	Soil A			Soil B			Control			Soil A		Soil B		Control	
	NH ₃	N ₂ O ₃	N ₂ O ₅	NH ₃	N ₂ O ₃	N ₂ O ₅	NH ₃	N ₂ O ₃	N ₂ O ₅	N ₂ O ₃	N ₂ O ₅	N ₂ O ₃	N ₂ O ₅	N ₂ O ₃	N ₂ O ₅
1															
2															
3															
4															
5															
6															

- (j) How do these organisms differ from those already studied, viz: those that grew on the soil, dust and milk plates, in regard to the nature of their food supply?
- (k) In what part of a farm sewage disposal system consisting of a septic tank and drains, do these organisms function?
- (l) In large municipal sewage disposal plants, how are these organisms helped in their work by special mechanical devices?

Exercise 18. Denitrification

MATERIALS:

- 5 tubes of Giltay's solution
- 1 fermentation tube of nitrate broth
- Soil samples
- Horse manure
- A culture *Ps. pyocyaneus*

If there is an accumulation of nitrates in the soil and anaerobic conditions are established by the soil becoming saturated with water, in the presence of large amounts of organic matter loss of nitrogen may result. This loss of nitrogen is due to the action of anaerobic organisms which reduce nitrates.

The nitrates may be reduced to nitrites only, or completely to free gaseous nitrogen. In the latter case there is an actual loss of soil fertility.

- 1. Inoculate 5 tubes of Giltay's solution as follows:
 - 1 tube with soil A
 - 1 tube with soil B
 - 1 tube with horse manure
 - 1 tube with a pure culture of *Pseudomonas pyocyaneus*
 - 1 tube leave sterile and uninoculated and label "sterile control."

GILTAY'S SOLUTION

Water	1000.00	grams
Potassium nitrate (KNO ₃)	2.00	"
Asparagin (C ₄ H ₈ N ₂ O ₃)	1.00	"
Magnesium sulphate (MgSO ₄)	2.00	"
Calcium citrate (Ca ₃ (C ₆ H ₅ O ₇) ₂ + 4H ₂ O)	8.00	"
Monobasic potassium phosphate (KH ₂ PO ₄)	2.00	"
Calcium chloride (CaCl ₂)	0.20	"
Iron chloride (FeCl ₂)	0.20	"

- 2. Inoculate the fermentation tube of nitrate broth with horse manure. Use about one gram.
- 3. Place all the cultures in the incubator for two days or one week.
- 4. Test the test tube cultures first for nitrites, and if nitrites are absent, then test them for nitrates.
- 5. Test the fermentation tube culture in the same way and if gas is present determine its composition as was done in Exercise 12.
- 6. Record the results on the regular note paper as shown in table V.
 - (a) What changes have taken place in the fermentation tube culture?
 - (b) What has happened?
 - (c) How are the changes to be explained?
 - (d) What changes do the tests indicate?
 - (e) Under what kind of conditions did the changes come about?
 - (f) In Giltay's solution, what substances are food for energy and what food for growth?
 - (g) In the main, what are the changes produced in Giltay's solution by the bacteria?
 - (h) Where do the organisms get their oxygen?

TABLE V.—Denitrification by Soil and Manure Bacteria

Source of bacteria	Medium	Gas Formation	Nitrites	Nitrates
Soil A				
Soil B				
Horse manure				
Denitrifying organism				
Sterile control				

(i) How do conditions for denitrification come about in agricultural practice?

Exercise 19. Free Nitrogen Fixation

Non-symbiotic Type

MATERIALS:

- 2 one-fourth pint milk bottles and caps
- 25 cc. graduate
- Nitrogen-free liquid medium
- Soil samples
- Pure culture of *Azotobacter*

Many soils upon which legumes are not growing, and to which nitrogen is not added in the form of chemical compounds or fertilizers of any kind, still have the power of materially increasing their nitrogen content. This increase is due to the presence in the soil of species of free nitrogen-fixing bacteria. These organisms are capable of maintaining an existence and of fixing nitrogen independent of higher plants.

The organisms that have the power to take the free nitrogen of the air and combine it in their cells can grow in a nitrogen-free culture solution. This culture medium contains only inorganic salts in solution and some simple carbohydrate or similar carbon compound which contains no nitrogen, e. g., the sugars, some of the alcohols, as mannite, etc. Such a solution is prepared as follows:

Mannit ($C_6H_8(OH_6)$)	15.00	grams
Magnesium sulphate ($MgSO_4$)	0.20	"
Sodium chloride ($NaCl$)	0.20	"
Calcium sulphate ($CaSO_4$)	0.10	"
Calcium carbonate ($CaCO_3$)	5.00	"
Dibasic potassium phosphate (K_2HPO_4)	0.20	"
Water	1000.00	"

1. The above culture medium is on the desk. With the graduate place 25 cc. of it in each of the two milk bottles. Be sure to stir the medium well before measuring any of it, as the insoluble substances settle to the bottom.

2. Label the bottles. Inoculate one with two or 3 measures of soil A and the other with the same amount of soil B. Cap the bottles.

3. Place the bottles in the incubator at $25^\circ C$. where they will not be disturbed. Watch for the development of a white, gelatinous film which is very conspicuous and soon turns brown or black.

4. Make a microscopic preparation from the film on the surface of each solution, according to the following method: Place a drop of strong iodine solution upon a clean slide. Place some of the organisms from the film in the iodine solution. Carefully cover with a cover glass. The organisms will float in the solution under the cover glass.

5. Examine the preparation with the 4 m. m. objective. The large oval or coccoid bodies are some species of the free nitrogen-fixing organism, *Azotobacter*. Long, rather oval or mace-shaped organisms, which have a blue appearance, are sometimes seen; they are the anaerobic species of free nitrogen-fixing organisms, *Clostridium butyricum*.

6. Examine the agar slope culture of *Azotobacter* furnished. Draw and describe it according to the outline for study of species. See Appendix A.

7. Make one microscopic preparation from the agar and stain the organisms with methylene blue. Draw and describe the organisms as seen with the oil-immersion objective.

8. The relative number of these organisms in soils A and B can be judged by the rate and extent of the development of the brown film.

- (a) What is the shape of the *Azotobacter*?
- (b) Is the organism motile?
- (c) How does it compare in size with other bacteria?
- (d) What organisms have you studied of which it reminds you?
- (e) Is the organism aerobic or anaerobic?
- (f) What is the value of *Azotobacter* to the soil?
- (g) Where did the nitrogen come from that is in the film on the liquid in the bottles?
- (h) What use does *Azotobacter* make of the mannite?
- (i) What purpose is served by the calcium carbonate?

Exercise 20. Effect of Variation in Food on the Growth of *Azotobacter*

MATERIALS:

- 2 tubes of nitrogen-free agar
- 2 tubes of same agar without sucrose
- 2 tubes of the same agar with lactose in place of sucrose
- 2 tubes of the same agar without phosphate
- 1 9 c. c. sterile water blank
- Pure culture of *Azotobacter*

1. Prepare a heavy suspension of organisms from the pure culture by transferring them to the sterile water. Shake well until they are evenly distributed.
2. After carefully labeling the agar slopes, inoculate each one by making a single streak along the surface of the agar with a loopful of the suspension.
3. Incubate the cultures for a period of two weeks.
4. Keep careful watch of the cultures and record the extent and color of the growth on the various media.
 - (a) What food supply is best for *Azotobacter*?
 - (b) Why is some soluble carbohydrate so important?
 - (c) Which one of the compounds used appears to be the most essential for *Azotobacter*?
 - (d) What are the essential elements for most bacteria?

Exercise 21. Free Nitrogen Fixation
Symbiotic Type

MATERIALS:

- Preserved specimens of legume nodules
- Young leguminous plant with nodules
- 3 sterile Petri dishes
- 3 tubes nitrogen-free agar
- 3 tubes sterile water
- Sterile pipettes
- Pair of forceps
- Sterile knife
- Pure culture of *Rhizobium radicicolum*

Leguminous plants, such as clovers, peas, beans, lupines, and vetches, when grown in moist soils bear numerous nodules or tubercles upon their roots. At certain times in the plant's growth these nodules contain myriads of bacteria. These nodule-forming organisms can fix the free nitrogen of the air, in which state it becomes available to the plant. Such a relation existing between the bacteria and the plant is one of co-operation.

1. Remove a root from a fresh, green, leguminous plant with a nodule attached. Select as large a nodule as possible.
2. Carefully wash the nodule in three changes of sterile water.
3. Treat the nodule in 70 per cent alcohol for 3 to 5 minutes. Pour off the alcohol, and wash the nodule in three changes of sterile water.
4. Sterilize a clean slide by heating thoroughly in the flame.
5. When the slide is cool, place a large drop of water on it; and crush the nodule, with a sterile knife or scalpel in the water.
6. Label three Petri dishes 1, 2, 3 and by means of sterile pipette, place a large drop of sterile water in each.
7. By means of the sterile loop transfer one loopful of the milky material on the slide to the first Petri dish. Mix well. Sterilize the loop and transfer two loopfuls from the first Petri dish to the second. Sterilize the loop, and transfer two loopfuls from the second dish to the third. Mix well.
8. Prepare a slide for staining with a loopful of the material on the sterile slide.
9. Melt three tubes of legume agar, cool to 45°C. and pour the three plates. When the agar is solid, place the plates inverted, in moist chambers at 28° C. for ten days or two weeks. The nitrogen-free medium known as legume agar has the following composition:

Mannit (C ₆ H ₈ (OH) ₆)	-----	15 grams
Dibasic potassium phosphate(K ₂ HPO ₄)	----	0.20 “
Magnesium sulphate (MgSO ₄)	-----	2.20 “
Sodium chloride (NaCl)	-----	0.20 “
Calcium sulphate (CaSO ₄)	-----	0.10 “
Calcium carbonate (CaCO ₃)	-----	5.00 “
Water	-----	1000.00 “
Agar	-----	15.00 “

10. When dry, fix the preparation made from the crushed nodule, and stain in dilute (1:5) fuchsin for three minutes.

11. Examine, draw and describe the plant on the desk. Leave the roots in the water for this purpose. Carefully examine the nodules. Note their shape, color, position, and size.

12. Examine the stained preparation made from the nodule. Look very carefully for irregular shaped cells: pear, club, Y and T-shaped individuals. It is quite possible that all of these shapes may not be seen in the same preparation. The bacteria when appearing in these shapes are called *bacteroids*. Draw and describe the organisms. See instructor's demonstration.

13. Draw and describe typical nodules from the preserved specimens of red clover, alfalfa, soybeans, garden peas, and lupine.

14. Prepare a slide from one of the colonies marked by the instructor and examine the organisms as before.

(a) Are there any bacteroids?

(b) What kind of organisms are present?

15. Examine the pure cultures of *Rhizobium radicicolum* furnished. Draw and describe the culture according to the outline of the study of cultures.

16. Examine the organisms after staining them with crystal violet or carbol fuchsin. Be sure that some of the gelatinous growth is on the slide. The organisms will appear unstained on a stained background.

17. Draw and describe the organisms as seen with the oil-immersion objective.

18. (a) What was the shape of the organisms as seen in the nodule?
As seen from the colony? As seen from the streak culture?

(b) What type of structure was revealed in the organisms from the nodules?

(c) What are bacteroids?

(d) What is the difference between the organisms in the nodules and those in the pure cultures?

(e) What relation do these organisms bear to the nitrogen supply of legumes?

(f) Why are legumes such good crops for the land?

(g) What is meant by inoculation of legumes?

(h) When is inoculation necessary?

Exercise 22. Cellulose Fermentation

MATERIALS:

- 2 20 c.m. test tubes
- 100 c.c. of Omeliansky's solution.
- A suspension of cow manure
- 4 strips of filter paper
- 25 c.c. graduate
- Vaseline

Cellulose is the main constituent of the woody fiber of all plant tissue. In soils, marshes, the bottoms of ponds and rivers, manure piles, and compost heaps, cellulose is constantly undergoing decomposition. The purest forms of cellulose are quickly attacked by bacteria, and the progress of the decomposition of a substance like filter paper is easily observed.

Ammonium sulphate ($\text{NH}_4)_2\text{SO}_4$)-----	0.5 grams
Magnesium sulphate (MgSO_4)-----	0.5 “
Di-basic potassium phosphate (K_2HPO_4)---	1.0 “
Calcium carbonate (CaCO_3)-----	5.0 “
Sodium chloride (NaCl)-----	a trace
Water-----	1000.0 “

This solution is called Omeliansky's solution.

1. Fill two large test tubes one-third full of the culture solution on the desk. See that the solution is well agitated before taking any of it, as the insoluble substances settle out.
2. Add about 5 cc. of a suspension of cow manure to each tube.
3. Immerse two strips of filter paper in a vertical position.
4. Cover the surface of the liquid with about one-half inch of melted vaseline to exclude air.
5. Sterilize one of the tubes to prevent bacterial growth. Observe the precautions of Exercise 7, when labeling the tubes.
6. Incubate both tubes at 37° C.
7. Compare the tubes after changes in the paper are apparent.
8. Draw and describe the appearance of the paper.
9.
 - (a) What is the odor like in the tubes?
 - (b) What is the origin of the odor?
 - (c) How has the color of the paper and deposits changed?
 - (d) Chemically what kind of a substance is filter paper and what does it yield on hydrolysis?
 - (e) What are the by-products of cellulose fermentation? What service do they render in the soil?
 - (f) Why is an abundance of cellulose necessary in the heating of manure?
 - (g) What kinds of manure heat?
 - (h) Give three additional instances where cellulose fermentation is important in farm practice?

Exercise 23. Hydrogen Sulphide Formation from Protein

MATERIALS:

3 tubes of egg gelatin

3 tubes of egg agar

Garden soil

Cultures of: *Escherichia coli*
Bacillus mycoides
Bacillus graveolens

1. Observe the precautions in handling gelatin, and prepare a stab culture of each of *Escherichia coli*, *Bacillus mycoides* and *Bacillus graveolens*.

2. Incubate the cultures at 20° C.

3. Melt the agar and add about 0.1 gram of sterile lead carbonate to each tube. Mix well by rolling the tubes between the hands.

4. Inoculate one tube, after cooling to 45° C. with a loopful of garden soil. Mix well. Inoculate the second tube with one loopful from the first. Mix well. Inoculate the third tube from the second, using three loopfuls.

5. When the agar is solid incubate the culture at 30° C.

6. After one week examine the agar shake cultures for black colonies, and the gelatin stab cultures for liquefaction.

7. If the gelatin has liquefied, notice the odor and add a few drops of 5 per cent lead acetate solution to each tube. Observe the results?

- (a) What is the black substance formed upon the addition of lead salts?
- (b) What is the source of the hydrogen sulphide?
- (c) How do you explain the power of these organisms to liquefy gelatin and produce "lead blackening"?
- (d) From what compound in the media is the H_2S produced?
- (e) Where do these organisms occur in nature?
- (f) What is the practical value of these organisms?

Exercise 24. Sulphate Reduction

MATERIALS:

100 cc. of sulphate medium

3 tubes of sulphate agar

One two ounce bottle with cork and string

Old sewage

SOLUTION FOR SULPHATE REDUCTION

Di-basic potassium phosphate (K_2HPO_4)	--	0.5 grams
Sodium lactate ($NaC_3H_5O_3$)	-----	0.5 "
Ammonium sulphate ($(NH_4)_2SO_4$)	-----	2.0 "
Ferrous sulphate ($FeSO_4 + 7H_2O$)	-----	trace
Tap water	-----	1000.0 cc.

1. Fill the bottle to the neck with the solution and inoculate it with one cc. of sewage. Stopper tightly and tie in the cork.
2. Melt the tube of sulphate agar and cool it to 45 degrees C. Inoculate it with a few loopfuls of sewage. Mix well and solidify quickly in cold water.
3. Incubate the cultures at 30° C. for one week.
4. Examine the cultures and uncork the bottle. Describe the changes.
5.
 - (a) What causes the change in color?
 - (b) What is the odor?
 - (c) Under what kind of conditions as to oxygen did the change take place?
 - (d) What are the chemical changes?
 - (e) What other process studied was similar to this?

PART IV

DAIRY BACTERIOLOGY

Exercise 25. The Bacteriological Examination of Milk

MATERIALS:

- 11 tubes of laetose agar
- 11 sterile Petri dishes
- 3 99 c.c. water blanks
- 4 9 c.c. water blanks
- Case sterile pipettes
- Sample of high-grade milk
- Sample of market milk

Milk that is to be examined must be well mixed. Creaming and settling have some effect upon the distribution of bacteria in milk.

FIG. 17—MANNER OF MAKING DILUTIONS OF HIGH GRADE MILK

FIG. 18—DIAGRAM OF THE DILUTIONS FOR ORDINARY MARKET MILK

Samples collected for bacteriological examinations are usually taken in sterile, wide-mouthed, glass-stoppered bottles, packed in ice, and examined as soon as possible.

Ordinary milk contains so many bacteria that it has to be diluted greatly in order that such a number of colonies will be present on the plates, that the counting may be done easily and accurately and that the results may be of value.

1. Prepare lactose-agar plates from high-grade milk and ordinary market milk. Make duplicate dilutions according to the outline in Figs. 17 and 18. Be sure that all dilutions are well shaken. Make duplicate plates of the high grade milk with 1/1000 cc. and 1/10,000 cc. and of the market milk with 1/1000 cc., and 1/10,000 and 1/100,000 cc. Make one blank for a control.

2. Label each dish with the kind of milk, dilution, and the desk number before making the dilutions.

3. Melt the agar and cool it to 45 degrees C. before pouring the plates.

4. Place the plates, inverted, in the incubator for 48 hours or one week at room temperature.

5. Count the colonies, using a plate counter if necessary. When giving the final count per cc. of the milk keep in mind the number of colonies allowable on agar plates. Arrange the results in tabular form as shown in Table VI. Place the table on the regular note paper.

TABLE VI.—Bacterial content of milk

Name of sample	Dilution	Colonies on plate		Bacteria per cc.	Number of kinds	Remarks
		I				
		II				
		I				
		II				
		I				
		II				
		I				
		II				
		I				
		II				

6.
- (a)

How do the total number and the kind of colonies compare in the two milk samples?
- (b)
- Does each colony represent a single organism?

(c)

- (d) How would you make plates containing $1/1,000,000$ and $1/10,000,000$ cc. of milk.
- (e) Why not use 0.1 cc. in preference to 1.0 cc. for the inoculation of the plates?
- (f) What sometimes causes colonies to be in a mass in the center of a dish or in a mass near one side?
- (g) Where does the moisture come from that condenses on the covers of the dishes?
- (h) How does the total number of bacteria in milk compare with the number in soil?
- (i) Why is it not desirable to average the counts of all the plates in getting the final results?
- (j) What are spreading colonies? In what way do they affect plate counts?

Exercise 26. Milk Contamination

MATERIALS:

- 10 tubes of lactose agar
- 10 sterile Petri dishes
- 1 tube of sterile milk
- 3 9cc. water blanks
- 4 99cc. water blanks
- Case of pipettes
- Sample of aseptic milk
- Cow hairs
- A 1 to 1000 suspension of manure
- Small pail containing skimmed milk

PART I.

Contamination from the Interior of the Udder, Aseptic Milk,

The milk upon the desk was procured as follows: The cow's udders were carefully cleaned. The milker's hands were carefully washed in a disinfectant and the milk drawn in as nearly a dust free atmosphere as possible. The first streams of milk were thrown away and then the milk was drawn as carefully and quickly as possible into a sterile flask. In this manner the danger from outside contamination was reduced to the minimum.

1. Melt two tubes of lactose agar and cool to 45° C. Dilute 1 cc. of the milk in a 9 cc. water blank and prepare one lactose agar plate, using 1 cc. of the dilution. Dilute 1 cc. of the milk in a 99 cc. water blank. Prepare a lactose agar plate, using 1 cc. of this dilution. Be sure that each one of the plates is labeled with the dilution, name of the milk, and the desk number. Incubate the plates, inverted, in the incubator one week.
2. Set the sample of milk away in the locker and allow it to incubate for the same length of time as the plates.
3. Observe the number of colonies that have developed on the plates. Tabulate the results on the regular note paper as in Table VII.

Table VII.—Bacterial Contents of Aseptic Milk

Dilution	Colonies on plate	Bacteria per cc.	Number and types of kinds

PART 2.

Contamination from Cow Hairs

1. Melt a tube of lactose agar. Pour the agar into a Petri dish and allow it to solidify.
2. When the agar is solid, with sterile forceps carefully remove two or three cow hairs from the dish upon the desk and place them upon the surface of the agar. Make sure that the hairs are in contact with the surface of the culture medium for their entire length. Press them down with the sterile loop. Be careful not to break the surface of the medium.

3. After the plate has incubated in the desk from four to six days, examine it. Observe the kinds of bacteria and the relative number of each. Distinguish the molds from the bacteria. Draw the hair with the bacteria and mold growths.
4. Drop a cow hair into a tube of plain sterile milk. Leave the tube in the incubator from four to six days. Note the kind of fermentation that takes place.

PART 3
Contamination from Manure

1. There is a suspension of one gram fresh cow manure in 999 cc. of sterile water on the desk.
2. Place 1 cc. of the 1/000 manure suspension in a 99 cc. water blank and place 1 cc. of this dilution in each of the two Petri dishes, using a sterile pipette. With another sterile pipette place 1 cc. of the 1 to 100,000 dilution in a 9 cc. water blank and use 1 cc. of it for each of the two plates with a dilution of 1 to 1,000,000.
3. When the agar is melted, cool it to 45° C. and pour the four plates. Place them in the incubator for 48 hours.
4. Observe the different kinds of colonies. Notice the relative number of the different kinds.
5. Count the colonies and estimate the number of organisms per gram of manure. Tabulate the results on the regular note paper as in Table VIII.

PART 4.
Contamination from Utensils

Dilution	Colonies Per plate	Bacteria per gram			Number and types of kinds
1/100,000 gm.	I	I			
	I	II			
1/1,000,000 gm.	I	I			
	II	II			

1. Pour the milk out of the pail to be found on the desk and rinse the pail well with tap water. Drain as dry as possible.
2. Pour in 100 cc. of sterile water, close and shake well.
3. Place 1 cc. of this wash water in a sterile Petri dish.
4. Rinse the can with a cupful of boiling hot water and drain dry.
5. Pour in 100 cc. of sterile water, close and shake well.
6. Place one cc. of this wash water in a sterile Petri dish.
7. Boil 500 cc. of water in the can for 5 minutes.
8. Place 1 cc. of this water in a Petri dish.
9. Be sure that the Petri dishes are labeled with the number of the washing and desk number. Use lactose agar and pour each plate.
10. Place the inverted plates in the incubator for 48 hours.
11. Tabulate the results on the regular note paper, showing the relation of bacterial content of washings to treatment. See Table IX.

TABLE IX.—Reduction of Bacteria in Milk Utensils Due to Heating

Treatments	Bacteria per cc. of wash water	Types of bacteria
1		
2		
3		

- (a) Are there many kinds of colonies on the aseptic milk plates?
- (b) Did any changes take place in the milk? What was the nature of the change if any?
- (c) What happened in the case of the cow hair in milk?
- (d) If the moisture content of manure is 80 per cent, what is the bacterial content per gram of the dry manure?
- (e) How does the total number of bacteria in manure compare with the number in milk and soil?
- (f) If 0.56 gram of dry manure falls into each gallon of milk from a dirty cow, how many organisms per cc. will be introduced into 20 pounds of milk from the same cow? 1 quart=0.946 liter=2.08 pounds.
- (g) How can the contamination of milk from milk utensils be controlled?
- (h) Which treatment was most efficient?
- (i) What construction in pails is best?
- (j) How are pails and utensils usually treated?
- (k) How is sterilization in good dairies usually accomplished?
- (l) Name the most important sources of milk contamination in order of their importance?

Exercise 27. Milk Fermentation

Acid Fermentation Caused by *Streptococcus Lactis*

MATERIALS:

- a. 1 tube of plain broth
- b. 1 Durham tube of lactose broth
- c. 1 Durham tube of saccharose broth
- d. 1 Durham tube of glycerin broth
- e. 1 fermentation tube of dextrose broth
- f. 1 lactose agar slope
- g. 3 tubes of lactose agar for plates
- h. 1 tube lactose gelatin
- i. 1 small tube of plain milk
- j. 1 small tube of litmus milk
- k. 1 large tube of plain milk
- l. 4 sterile Petri dishes
- m. Ice water for gelatin
- n. Culture of *Streptococcus lactis*
- o. Outfit for determination of acidity

The souring or formation of acid in milk is the most common change observed in it and may be caused by a great variety of bacteria. The souring of milk without the formation of gas and with the production of the pleasant buttermilk flavor and odor is due to a group of bacteria of which *Streptococcus lactis* is the principal representative.

1. Label each of the culture tubes and the Petri dishes with the number of the exercise and the letter indicating the kind of culture medium.

2. Prepare loop dilution plates for colony formation. Label sterile Petri dishes number 1, 2, and 3. Melt three tubes of lactose agar and cool them not lower than 45° C. Label one tube number 1; one, number 2; and the remaining one, number 3. Transfer one loopful of the culture to the agar tube number 1. Mix the organisms and agar well. Transfer 3 loopfuls of the agar and organisms from tube number 1 to tube number 2. Mix well. Transfer 4 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.

3. Make a stab culture in the gelatin.

4. Inoculate all of the remaining tubes of media by means of the wire loop.

5. Incubate the gelatin culture in the ice box. Incubate all of the other cultures 5 to 7 days at room temperature.

6. Record the morphological, cultural, and biochemical properties of the organisms according to the outline in Appendix A. The description should be recorded on the regular note paper, following the order given below:

A. Physiology

1. Relation to Oxygen: aerobic, anaerobic, facultative.
2. Acid production: from dextrose, lactose, saccharose, glycerin.
3. Gas Production: from dextrose, lactose, saccharose, glycerin.
4. Production of pigment: fluorescent, violet, blue, green, yellow, orange, red, brown, pink.

B. Cultural Characteristics**1. Gelatin Stab.** *Draw the culture.*

- (a) Growth: *uniform, best at top, best at bottom.*
- (b) Line of puncture: *filiform, beaded, papillate, villous, arborescent.*
- (c) Liquefaction: *none, crateriform, napiform, infundibuliform, saccate, stratiform.*

2. Agar Slope. *Draw the culture.*

- (a) Growth: *scanty, moderate, abundant, none.*
- (b) Form of growth: *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
- (c) Elevation of growth: *flat, effuse, raised, convex.*
- (d) Lustre: *glistening, dull.*
- (e) Topography: *smooth, contoured, rugose.*
- (f) Optical Characters: *opaque, translucent, opalescent, iridescent.*
- (g) Chromogenesis: *-----photogenic, fluorescent.*
- (h) Odor: *absent, decided, resembling-----*
- (i) Consistency: *butyrous, viscid, membranous, brittle.*
- (j) Medium: *grayed, browned, reddened, blued, greened.*

3. Broth Culture.

- (a) Surface growth: *ring, pellicle, flocculent, membranous, none.*
- (b) Clouding: *slight, moderate, strong, transient, persistent, none, fluid, turbid.*
- (c) Odor: *absent, decided, resembling-----*
- (d) Sediment: *compact, flocculent, granular, flaky, viscid, on agitation, abundant, scant, none.*

4. Agar Colonies. *Draw a colony.*

- (a) Growth: *slow, rapid.*
- (b) Form: *punctiform, circular, irregular, myceloid, filamentous, rhizoid.*
- (c) Surface: *smooth, rough, concentrically ringed, radiate.*
- (d) Elevation: *flat, effuse, raised, convex, pulvinate, umbinate.*
- (e) Edge: *entire: undulate, lobate, erose, filamentous, curled.*
- (f) Internal structure: *amorphous, finely, coarsely-granular, filamentous, curled, concentric.*

5. Milk Culture. *Draw the litmus milk culture.*

- (a) Reaction to litmus.
- (b) Curd: *hard or soft; solid or porous.*
- (c) Whey: *little or abundant; clear or turbid.*
- (d) Digestion of curd: (Examine the plain milk culture).
- (e) Odor.
- (f) If curd has not formed, will it form on heating the culture to 100° C? Use tube I.
- (g) Percentage of acid formed.

Note: Titrate 10 cc. of the milk from tube K with N/10 NaOH and using phenolphthalein as an indicator. Calculate the per cent of acid using the following formula:

$$\text{Per cent acid} = \frac{\text{cc. N/10 NaOH} \times 0.009 \times 100}{\text{cc. milk used.}}$$

C. Morphology

1. Vegetative Cells.

- (a) Form: *spheres, short rods, long rods, filaments, commas, short spirals, long spirals, curved.*
- (b) Arrangement: *single, pairs, chains, fours, cubical pockets.*
- (c) Limits of size-----size of majority.
- (d) Ends: *rounded, truncate, concave.*

2. Capsules.

- (a) How stained-----

3. Motility.

4. Endospores: *present, absent.*

- (a) Location of endospores: *central, polar.*
- (b) Form: *spherical, elliptical, elongated.*

5. Staining Reactions.

- (a) Stain a preparation from the agar slope with carbol fuchsin.
- (b) Stain a preparation from the litmus milk culture with methylene blue.

- 6.
 - (a) Judging from the results obtained with the different kinds of media, under what conditions does *Streptococcus lactis* grow best?
 - (b) What is the texture and flavor of milk soured by this organism?
 - (c) Of what value is it in the dairy industry?
 - (d) Does the organism produce spores?
 - (e) Is all of the sugar of the milk fermented? If not, why not?
 - (f) What is the best temperature for the growth of the *Streptococcus lactis* group of organisms?

Exercise 28. Milk Fermentation

Acid Fermentation Caused by *Aerobacter aerogenes* group

MATERIALS:

- a. 1 tube of plain broth
- b. 1 Durham tube of lactose broth
- c. 1 Durham tube of saccharose broth
- d. 1 Durham tube of glycerin broth
- e. 1 fermentation tube of dextrose broth
- f. 1 lactose agar slope
- g. 3 tubes lactose agar for plates
- h. 1 tube gelatin
- i. 1 small tube of plain milk
- j. 1 small tube of litmus milk
- k. 1 large tube of plain milk
- l. 4 sterile Petri dishes
- m. Ice water for gelatin
- n. Culture of *Aerobacter aerogenes*
- o. Outfit for determination of acidity

A number of acid-forming bacteria also produce gas and undesirable flavors and odors in milk. On account of the second characteristic, their presence is very undesirable in milk, especially in that which is to be used for the manufacture of butter and cheese.

One of the organisms occurring in the intestines of cattle produces acid and gas from most sugars. This organism is *Aerobacter aerogenes*. Because manure so frequently gets into milk, most milk under favorable temperature conditions will show gas formation.

1. Label each of the culture tubes and the Petri dishes with the number of the exercise and the letter indicating the kind of culture medium.

2. Prepare loop dilution plates for colony formation. Label 3 sterile Petri dishes 1, 2, and 3. Melt three tubes of lactose agar and cool them not lower than 45° C. Label 1 tube number 1, one number 2, and the remaining one number 3. Transfer one loopful of the culture to the agar tube number 1. Mix the organisms and agar well. Transfer 3 loopfuls of the agar and organisms from tube number 1 to tube number 2. Mix well. Transfer 4 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 in 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.

4. Make a stab culture in the gelatin by thrusting the inoculated straight wire into the center of the solid medium nearly to the bottom of the test tube. Be sure the wire is perfectly straight.

5. Inoculate all of the remaining tubes of media by means of the wire loop.

6. Incubate the gelatin culture in the ice box. Incubate all of the other cultures 48 hours in the incubator.

7. Record the morphological, cultural, and biochemical properties of the organism according to the outline in Appendix A. The description should be recorded on the regular note paper following the order given below:

A. Physiology

1. Relation of Oxygen; aerobic, anaerobic, facultative.
2. Acid Production: from dextrose, lactose, saccharose, glycerin.
3. Gas Production: from dextrose, lactose, saccharose, glycerin.
4. Production of Pigment: fluorescent, violet, blue, green, yellow, orange, red, brown, pink.

B. Cultural Characteristics

1. Gelatin Stab. *Draw the culture.*
 - (a) Growth: *uniform, best at top, best at bottom.*
 - (b) Line of puncture: *filiform, beaded, papillate, villous, arborescent.*
 - (c) Liquefaction: *none, crateriform, napiform, infundibuliform, saccate, stratiform.*
2. Agar Slope. *Draw the culture.*
 - (a) Growth: *scanty, moderate, abundant, none.*
 - (b) Form of growth: *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
 - (c) Elevation of growth: *flat, effuse, raised, convex.*
 - (d) Lustre: *glistening, dull.*
 - (e) Topography: *smooth, contoured, rugose.*
 - (f) Optical characters: *opaque, translucent, opalescent, iridescent.*
 - (g) Chromogenesis: *-----photogenic. fluorescent.*
 - (h) Odor: *absent, decided, resembling.*
 - (i) Consistency: *butyrous, viscid, membranous, brittle.*
 - (j) Medium: *grayed, browned, reddened, blued, greened.*
3. Broth Culture.
 - (a) Surface growth: *ring, pellicle, flocculent, membranous, none.*
 - (b) Clouding: *slight, moderate, strong, transient, persistent, none, fluid, turbid.*
 - (c) Odor: *absent, decided, resembling-----*
 - (d) Sediment: *compact, flocculent, granular, flaky, viscid on agitation, abundant, scant, none.*
4. Agar Colonies. *Draw a colony.*
 - (a) Growth: *slow, rapid.*
 - (b) Form: *punctiform, circular, irregular, myceloid, filamentous, rhizoid.*
 - (c) Surface: *flat, effuse, raised, convex, pulvinate, umbonate.*
 - (d) Edge: *entire, undulate, lobate, erose, filamentous, curled.*
 - (e) Internal structure: *amorphous, finely-, coarsely-granular, filamentous, curled, concentric.*
5. Milk Culture. *Draw the litmus milk culture.*
 - (a) Reaction to litmus.
 - (b) Curd: *hard or soft, solid or porous.*
 - (c) Whey: *little or abundant; clear or turbid.*
 - (d) Digestion of curd: (Examine the plain milk culture).
 - (e) Odor.
 - (f) If the curd has not formed, will it form on heating the culture to 100° C? Use tube I.
 - (g) Percentage of acid formed. See Exercise 27.

C. Morphology

1. Vegetative Cells.

- (a) Form: *spheres, short rods, long rods, filaments, commas, short spirals, long spirals, curved.*
- (b) Arrangement: *single, pairs, chains, fours, cubical packets.*
- (c) Limits of size-----size of majority.
- (d) Ends: *rounded, truncate, concave.*

2. Capsules.

- (a) How stained-----

3. Motility.

4. Endospores: *present, absent.*

- (a) Location of endospores: *central, polar.*
- (b) Form: *spherical, elliptical, elongated.*

5. Staining reactions.

See Exercise 27.

6. (a) Is the organism Gram positive?

- (b) How does the per cent of acid compare with that produced by *Streptococcus lactis*.?
- (c) Does the organism produce spores?
- (d) How does the odor of the milk cultures compare with those fermented by *Streptococcus lactis*.?
- (e) What is the Wisconsin curd test?
- (f) What is its purpose?
- (g) Why are the curds kept at a high temperature?
- (h) Why is rennet added to the milk to be tested?
- (i) Do the bacteria remain in the curd or pass into the whey?
- (j) Why is dirty milk objectionable for butter and cheese making?
- (k) In what way does the action of *Aerobacter aerogenes* upon milk differ from that of *Streptococcus lactis*?
- (l) How is *Aerobacter aerogenes* related to the quality of milk?
- (m) What is the habitat of *Aerobacter aerogenes*?

Exercise 29. Milk Fermentation

Acid Fermentation Caused by *Lactobacillus Bulgaricus* Group

MATERIALS:

- Raw skimmed milk
- 1 125 cc. bottle, cork, and string
- Outfit for determination of acidity in milk

There are present in ordinary milk acid-forming bacteria which develop only under certain conditions. If milk is kept tightly stoppered to prevent mold growth, and held at a comparatively high temperature, it will become very acid due to the growth of certain bacteria usually termed the *Lactobacillus Bulgaricus* group.

- 1. Fill a 125 cc. bottle to the neck with raw skimmed milk. Cork tightly and secure the cork with a string as shown by the instructor. Label the bottle. Place in the incubator at 37° C.
- 2. At the end of 2, 7, and 14 days, open the bottle and examine the milk as to odor, acidity (by titration), and type of organism present. Microscopic preparations should be made after thorough shaking of the bottle. Some of the curd should be spread as thinly as possible on the slide. Methylene blue is a good stain to use.
- 3. Tabulate the observations on the regular note paper, showing the time, acidity, and morphology of the organisms present. See Table X.

TABLE X.—The Development of High-Acid Organisms in Milk

Incubation period	Drawing of organisms present	Acidity of milk
2 days		
7 days		
14 days		

- 4.
 - (a) How does the final acidity of the milk compare with that produced by *Streptococcus lactis* and *Aerobacter aerogenes*.
 - (b) What type of organism finally predominates?
 - (c) Is the flavor of the milk desirable or undesirable?
 - (d) What is the significance of the organism in fermented milk drinks?

- (e) Who advanced a theory of human longevity connected with this organism?
- (f) Are the organisms of this type of great practical importance? Explain the answer.
- (g) What agricultural industry makes use of this organism?

Exercise 30. Milk Preservation

MATERIALS:

- 6 sterile test tubes
- Sample of raw skimmed milk

It is very desirable to keep milk in as nearly the natural condition as possible until it is consumed. Bacteria are responsible for practically all of the changes that render milk undesirable for food purposes. With even the best methods of production, the contamination of milk is so great and the growth of bacteria so rapid that the milk becomes undesirable for food purposes in a short time. On this account methods for the preservation of milk are necessary.

1. Fill each of 3 sterile tubes one-third full of sweet milk.
2. Label the tubes and store one in the ice box, one in the desk and one in the incubator. Examine them at each laboratory period until the milk is curdled. Tabulate the time of curdling.
3. Fill 3 sterile test tubes one-third full of sweet milk.
4. Heat the tubes to 90° C. in water in the cup for 30 minutes.
5. Place one of the tubes in the ice box. Place one tube in the incubator. Permit the other tube to remain in the desk.
6. Examine the tubes after 24 and 48 hours. Tabulate the time of curdling on the regular note paper. See Table XI.

TABLE XI.—Effect of Temperature and Pasteurization on the Quality of Milk

Sample	Treatment	Time to curdle

7.
 - (a) In what order did the unheated tubes of milk curdle?
 - (b) What can you say of the temperature necessary to keep milk sweet for as long a time as possible?
 - (c) Why does it remain sweet under these conditions?
 - (d) Which tube of milk in the heated set showed signs of curdling or spoiling first?

- (e) Which tube remained "sweet" the longest?
- (f) What are the respective temperatures of the ice box, room, and incubator?
- (g) What can be done in storing milk in order to prolong its usefulness?

PART V.

BACTERIOLOGY OF WATER AND SEWAGE

Exercise 31. The Total Bacterial Content of Water

MATERIALS:

- 13 tubes of sodium caseinate agar
- 13 sterile Petri dishes
- Case sterile 1 cc. pipettes
- 1 sterile 10 cc. pipette
- 4 9 cc. sterile water blanks
- 1 99 cc. sterile water blank
- 3 dextrose broth fermentation tubes
- 2 samples of surface water
- 1 sample of ground water

1. Follows the dilution diagram (Fig. 19) and prepare plate cultures as follows: Lake water, 1/10 and 1/100 cc.; shallow well, 1 cc. and 1/10 cc.; ground water 1 cc. Make one "control" plate.

2. When the duplicate plates all contain their dilutions and the sterile "control" is made, melt the agar and cool it to 45° C. Pour the plates, mixing the water and agar well.

3. When the agar is solid, incubate the plates for one week at room temperature.

FIG. 19—DIAGRAM SHOWING DILUTIONS FOR WATER SAMPLES

4. Use one of the water samples designated by the instructor and inoculate the 3 fermentation tubes after labeling as follows: 10 cc., 1 cc. and 0.1 cc. The 0.1 cc. is obtained by taking 1 cc. of the 1 to 9 dilution.

5. Be careful that there are no air bubbles in the closed arm of the fermentation tube and incubate the cultures 48 hours at incubator temperature.

6. Count the colonies in the plate cultures and tabulate the results as shown in Table XII on the regular note paper.

TABLE XII.—Bacterial Content of Waters

Kind of sample	Dilution	Colonies per plate	Bacteria per cc.	Kinds of colonies	Gas from bacteria in		
					10 cc.	1 cc.	0.1 cc

7. Make a careful examination of the fermentation tubes for growth and gas formation.

Note: Each fermentation tube containing gas indicates the presence of a member of the common intestinal bacteria. The most commonly occurring forms are: *Escherichia coli* and *Aerobacter aerogenes*. On the assumption that at least one of the organisms is present in the tube, the approximate number in one cubic centimeter of water may be calculated.

- (a) What is the nature of the organisms developing from ground water as compared to those from surface water?
- (b) What quality of water is indicated by the results?
- (c) What chemical tests should go with these tests?
- (d) What is meant by the presumptive test for *Escherichia coli*?
- (e) What is the sanitary significance of *Escherichia coli* in water?

Exercise 32. The Bacteriological Examination of Sewage

MATERIALS:

- 7 tubes of sodium caseinate agar
- 7 sterile Petri dishes
- Case of sterile 1 cc pipettes
- 3 9 cc. sterile water blanks
- 3 99 cc. sterile water blanks
- Sample raw sewage

1. Prepare dilutions of the sewage according to the scheme in Fig. 20. Prepare duplicate plates with 1/100,000 and 1/1,000,000 cc. Make one control.
2. Melt the agar and cool it to 45° C. Pour the plates, and when the agar

FIG 20—MANNER OF MAKING SEWAGE DILUTIONS

is solid, incubate them at room temperature for one week.

3. Count the plates and record the data as in Table XIII.

TABLE XIII.—Baeterial Content of Sewage

Dilution	Colonies on plate	Bacteria per cc.		Kind of colonies
1/10,000 cc.	I			
	II			
1/100,000 cc.	I			
	II			
1/1,000,000 cc.	I			
	II			

- (a) How does the bacterial content of sewage compare with that of water?
- (b) What kinds of organisms predominate in raw sewage?
- (c) What chemical changes take place in sewage?

- (d) Give the most important reasons for not discharging raw sewage into surface waters.
- (e) Why are the same culture media used for water and sewage examination as for the examination of soils?
- (f) What is meant by the phrase "sewage disposal?"
- (g) What takes place in sewage during biological disposal?

Exercise 33. The Purification of Water

MATERIALS:

Sterile Berkefeld filter for faucet
0.003 per cent of solution of chloride of lime
1 sterile test tube
5 sterile Petri dishes
5 tubes of sodium caseinate agar
Case of sterile 1 cc. pipettes

Many cities must use water which is subject to contamination with harmful types of bacteria. Such water must be rendered safe. To accomplish this, the water may be filtered slowly through sand filters; or by the addition of lime and an aluminum salt, a gelatinous precipitate can be produced in the water, and the bacteria will be held in the precipitate which can be removed by coarse filters. Chemicals which destroy bacteria and at the same time are not injurious to the consumer, are sometimes used to purify drinking water. Calcium hypochlorite is frequently used for this purpose.

1. Collect some tap water in a sterile test tube and prepare a plate culture with 1 cc of the water.

2. Add 1 cc. of a 0.003 per cent solution of chloride of lime to the 9 cc. of tap water in a sterile test tube and shake. This gives a solution of 1 part of chlorine in 1,000,000 parts of water. Permit the tube to stand 15 minutes and prepare two 1 cc. plate cultures from it.

3. Collect some tap water as it passes through a sterile Berkefeld filter. Prepare two 1 cc. plate cultures from it.

4. Incubate the cultures at room temperature for one week.

5. Examine the 5 culture plates; count and record the number of bacteria in the water before and after treatment.

- (a) What was the effect of filtering water and of treating it with hypochlorite?
- (b) Which method was the more efficient?
- (c) Which method is the more practicable for water supplies?
- (d) What are the objections to unglazed porcelain filters?
- (e) What is the principle upon which hypochlorite is supposed to work in destroying bacteria?
- (f) What is the practical objection to the hypochlorite treatment of water supplies?

PART VI.

BACTERIOLOGY OF ANIMAL DISEASES

Exercise 34. Preparation of General Disinfectant

MATERIALS:

A 20 per cent solution of corrosive sublimate (HgCl_2) in concentrated HCl

2 liter stone jar

Graduated cylinder

As many of the pathogenic organisms produce diseases in man, as well as in the lower animals, an efficient disinfectant must be used in the laboratory when working with pathogenic organisms.

A convenient disinfectant to use is corrosive sublimate or mercuric chloride (HgCl_2) in a 1 to 1,000 solution.

1. Prepare 1.5 liters of corrosive sublimate, 1 to 1,000 from the 20 per cent solution on the desk. The 20 per cent solution is made with concentrated commercial hydrochloric acid (HCl) because mercuric chloride is quite insoluble in water.

2. Dilute the strong solution at the rate of 5 cc. to 1,000 cc. of water which gives approximately a 1 to 1,000 solution. Make the dilution in the stone jar and rinse the graduate well, pouring the rinsings into the solution.

3. The solution is to be used on a sponge in wiping up the surface of the desk at the end of the laboratory period. If any material containing pathogenic organisms is accidentally spilled on the desk or floor, it should be covered at once with a cloth saturated with the mercuric chloride solution (1:1000).

(a) Give the places where mercuric chloride cannot be used and explain why.

(b) Is mercuric chloride a germicide or an antiseptic?

(c) Why must gold rings and other jewelry not come in contact with solutions of mercuric chloride.

Exercise 35. Bacteriology of Anthrax or Splenic Fever

MATERIALS:

3 plain agar slopes

1 plain broth

1 9 cc. water blank

Inoculated animal for post-mortem

Sections showing organisms

Slides

Eosin-methylene blue stain for blood

Culture of *Bacillus anthracis*

The discoverer of the cause of tuberculosis, Robert Koch, set down four laws or rules for the study and proof that any specific organism causes a specific disease. These are known as Koch's postulates and are: (1) The organism must be demonstrated in the circulation of tissues of the diseased animals; (2) organism must be isolated and cultivated in artificial media outside the body, and successive generations of a *pure culture* of that organism must be obtained: (3) such pure cultures must, when introduced into a healthy and susceptible animal, produce the specific disease; (4) the organism must be re-isolated from the blood or tissues of the inoculated animal.

It is true that there are some diseases in which the specific organism cannot be cultivated outside of the animal body, or for which the causal organism has not been discovered. Examples of such diseases are hog cholera and rabies. Such diseases do not fulfill postulates (2) and (3), yet by the majority they are **provisionally accepted** as caused by a specific organism. Anthrax is chosen as a typical pathogenic organism that fulfills all of the postulates of Koch.

Note.—Remember that the inoculating needles should be flamed **immediately** after they have been used.

1. From the material supplied, inoculate an agar slope and a tube of broth. Label the tubes with the name of the organism and the desk number.

2. Leave the tube from which you have inoculated your culture on the desk. Wipe off the desk with the HgCl_2 solution. Wash your hands with 1/1000 HgCl_2 solution, then with soap and water.

3. Draw and describe the cultures in broth and on the agar slope. Follow the outline in descriptions. See Appendix A.

4. Make microscopic preparations from the agar slope and from the broth, staining with carbol fuchsin and Gram's stain. The slides are to be labeled with the student's name, name of the organism, and kind of medium from which the specimen was taken, and the kind of stain used. **Remember that you are working with a dangerous organism and be especially careful not to scatter any of the organisms in making the slides.**

5. Draw and describe the preparations on each slide. See that the instructor examines and checks each one.

6. Observe the instructor inoculate a guinea pig with the same anthrax culture that has been used for study.

7. Clean up the desks as before.

8. Post-mortem upon the guinea pig inoculated at the previous period. Carefully observe the method of procedure in the post-mortem examination of animals and the location of the various internal organs.

9. With the sterile platinum loop inoculate an agar slope from the heart blood. Place it in the incubator for 48 hours. Make the culture at the post-mortem table, not at the desk. Make a similar culture from heart blood of a healthy pig killed by chloroform.

10. Clean four slides very clean. Label two heart blood, one spleen, and one liver.

11. At the post-mortem table make as thin smears as possible with the loop from the heart blood, spleen, and liver of the animal. Be sure the loop is sterilized after each operation and handle the slides with care. Make a slide from the heart blood of a healthy animal.

12. Permit the smears to air-dry. When perfectly dry, fix the smears of liver and spleen by heating in the usual way. Stain the liver and spleen smears by Gram's method.

13. Fix the heart blood smear by standing it in methyl alcohol from 10 to 15 minutes.

14. Transfer without washing to eosin for 5 or 10 minutes. Wash and blot dry.

15. Stain in aqueous methylene blue until the smear has a lavender or rose tint, usually about 3 to 5 minutes. Wash and dry.

16. Examine first with the 16 m. m. objective until a good field is found.

17. Examine with the oil-immersion objective. The red blood cells will be red. The nuclei of the white blood cells will be blue and their cytoplasm will be pink with red granules. The anthrax bacilli will be blue and the capsules will appear clear as if unstained.

18. Draw and describe a good field in the above preparation.

19. Examine the agar slope culture made from the heart blood. Draw and describe it. Is there any growth on the slopes inoculated from the blood of the healthy animal?

20. Prepare a slide from the above culture and stain it with carbol fuchsin. Draw and describe the organisms as seen under the oil-immersion objective.

21. Examine, draw and describe the organisms as seen in stained sections of tissue.

22. **Leave all of the cultures in a tumbler on the desk** in order that they may be sterilized.

23. (a) On what kind of media, liquid or solid, do spores form most rapidly and why?

(b) There are many organisms in soil and in the dust of the air that grow like anthrax and appear very much like anthrax in cultures? How could you prove such a culture was not anthrax?

(c) Why does anthrax produce a growth like a tuft of cotton in bouillon?

(d) Why is it so difficult to eliminate anthrax from an infected farm?

(e) What is the best method to prove positively that anthrax is the cause of the death of an animal?

Exercise 36. The Bacteriology of Tuberculosis

MATERIALS:

Museum specimens of tuberculous tissues

Sputum contain *Mycobacterium tuberculosis*

Fresh tuberculous tissues

Acid alcohol

1. Examine and describe the specimens of tuberculous tissues in the laboratory.

2. Make a uniform smear of some tuberculous sputum on a clean slide. Air dry and fix with gentle heat.

3. Stain the smear with hot carbol fuchsin for five minutes by placing the slide in a tube of the stain heated by boiling water. Wash off under the tap all of the stain that will come off. Decolorize the preparations in acid alcohol (80 per cent alcohol with 3 per cent nitric acid) until the alcohol that runs from the slides is no longer tinged with red. Wash in tap water. Stain the preparations one to three minutes with methylene blue. Wash in tap water. Dry and examine with the oil-immersion objective. The organisms will appear as very small red rods on a blue background. If the preparation is not satisfactory, repeat until a good one is obtained.

4. Examine tuberculous animals and prepare two smears on clean slides from tuberculous glands, liver or spleen. Grasp the tuberculous material in a pair of dissecting forceps and carefully grind, crush and smear it over the center of the slide. Dry the smear in the air and fix it in the usual manner. Stain in the same way as with sputum.

5. Draw a good field from each preparation, showing the organism and the surrounding tissue materials. Describe the organisms and note especially their size and shape.

- (a) What is meant by an "acid fast" organism?
- (b) Name some other acid-fast organisms besides the tubercle organism.
- (c) Where may they be found?
- (d) What parts of the body are most often affected in the case of tuberculosis of the cow?
- (e) What parts of the body may be affected?
- (f) What is meant by a calcified tubercle?
- (g) What is meant by open tuberculosis?
- (h) What is meant by closed tuberculosis?
- (i) How may the disease be acquired by cattle?
- (j) How may it be acquired by hogs?

Exercise 37. The Agglutination Test

MATERIALS:

Agar slant cultures of *Alcaligines abortus*
Physiological salt solution
Sterile hypodermic syringe
Diluted immune and normal rabbit serum
Graduated sterile 0.1 cc. pipettes
Case 1 cc. sterile pipettes
Rack for small test tubes
10 small test tubes
Rabbits

The agglutination test is a means of detecting one group of antibodies in the blood serum of animals naturally or artificially inoculated with bacteria. These substances are called agglutinins. The test is highly specific, and consists of a clumping of a suspension of the bacteria in salt solution when they are brought in contact with their specific antiserum. The content of the agglutinins in a given serum is determined by dilution of the serum with 0.85 per cent sodium chloride solution. The highest dilution of a serum which will agglutinate or throw the bacteria out of suspension determines the titer of the serum. The test may be made macroscopically in test tubes or microscopically with a hanging drop.

1. Immunize a rabbit against *Alcaligines abortus* as follows:

- (a) First inoculation. Inject, intraperitoneally 0.5 cc. of a suspension of the bacteria washed off an agar slope with salt solution.
- (b) Second inoculation. Five days after the first inoculation inject the animal as before with 1 cc. of a suspension of the same culture.
- (c) The third inoculation may be made five days after the second using a suspension of 3 cc. of the culture.

2. One week after the last injection the animal should be bled. A preliminary test may be made with a few drops of blood from an ear vein. In case the blood gives a sufficiently high titer, 1:100 or above, a larger quantity of blood may be drawn.

3. With the help of the instructor bleed the animal directly from the heart. About 8 or 10 cc. of blood should be taken with a sterile syringe. After drawing the blood is stored in a sterile test tube at ice box temperature, for 48 hours.

4. The clot, which soon forms, should be separated from the walls of the tube after 24 hours with a sterile glass rod. The contracting clot will squeeze out the serum, which should be clear and of a light yellow color. The clear serum should be removed with a sterile fine capillary pipette and stored in a sterile test tube for use.

5. Use fresh 4 days old cultures of *Alcaligines abortus* and prepare salt solution suspension as was done for the inoculation of the animal.

6. Prepare a stock solution of 1:10 from both immune and normal sera. This may be done by diluting 0.1 cc. of serum in 1.0 cc. of sterile salt solution. The other dilutions can be made from this stock dilution.

7. With a sterile 1 cc. pipette measure 1 cc. of the bacterial suspension (antigen) into each of eight small test tubes. Arrange the tubes in two rows in the rack. Number the tubes from left to right in the two rows as follows: Front row: A1, A2, A3, and A4; back row: B1, B2, B3, and B4. Place the immune serum in the front row of tubes.

FIG. 21—DIAGRAM FOR SERUM DILUTIONS

The dilutions are made with the 0.1 cc. pipette. The procedure shown for the immune serum is the same for the normal serum.

8. Use a sterile 0.1 cc. pipette, and prepare the following dilutions of each of the sera; rinsing the pipette in salt solution after each dilution. Mix the antigen and sera after each dilution by inversion over the index finger. Wipe off the tip of the finger with a clean cloth after each mixing. Transfers and dilutions, made according to the diagram (Fig. 21) will give in tube 1, 1:50; tube 2, 1:100; tube 3, 1:200; and tube 4, 1:500.

9. After the dilutions of the sera are made carefully wash the 0.1 cc. pipette in salt solution and then in distilled water.

10. Place the rack holding the tubes in the 37° C. incubator for 48 hours.

11. Remove the rack and tubes from the incubator. Examine the tubes by transmitted light. All tubes in which the bacteria have not settled out have not agglutinated. Clumps of bacteria floating in the solution are partly agglutinated.

12. Compare the dilutions made with the antiserum and with normal serum. Tabulate the results, by dilutions for the two sera. In the diagnosis of bovine contagious abortion agglutinations in dilutions of 1:50 and above indicate a positive test.

13.
 - (a) What is the name of the antibodies used in this exercise?
 - (b) Name two other kinds of antibodies?
 - (c) What important human disease may be diagnosed by means of this test?
 - (d) What name is applied to it when used for the above human disease?
 - (e) What is the value of such a test as this?
 - (f) What substances in the bacteria probably stimulate antibody production?
 - (g) How may this be related to the specificity of the serological tests?
 - (h) Of what value is such a test as this in the classification of bacteria?

Exercise 38. Test of Disinfectants

MATERIALS:

- A 10 per cent solution of carbolic acid
- A 1:500 solution of mercuric chloride
- 8 tubes of plain broth reaction pH 7.0.
- 1 tube of sterile distilled water
- 2 empty sterile test tubes
- 1 box of sterile 1 cc. pipettes
- Burettes for measuring disinfectants
- Culture of *Escherichia coli*
- Culture of *Bacillus anthracis*

Much misinformation is prevalent concerning the value of disinfectants. In order to gain some first-hand knowledge concerning some of the disinfectants in common use, two will be examined.

Each student will test one disinfectant. The disinfectants will be assigned by the instructor.

Escherichia coli will be used for example of a non-spore-forming organism and *Bacillus anthracis* will be used as an example of a sporogenous type.

1. Make an agar streak culture of *Escherichia coli* and of *Bacillus anthracis*. Place the cultures in the 30° C. incubator for 48 hours.

2. By means of a sterile 1 cc. pipette measure 4 cc. of sterile distilled water into each of the two sterile test tubes. Label one tube *Escherichia coli* and the other *B. anthracis*.

3. By means of the sterile loop prepare suspensions of the two cultures, transferring organisms from the agar streak cultures.

4. Using care to avoid contamination measure with a burette, 4 cc. of the disinfectant assigned, into each of the suspensions of bacteria. The dilution produced will allow a 5 per cent solution of phenol or a 1:1000 solution of mercuric chloride to act upon the bacteria.

TABLE XIV.—Exposure Table for Disinfectant Tests

<i>Escherichia coli</i>	<i>Bacillus Anthracis</i>
3 minutes	1 ½ hours
10 minutes	24 hours
30 minutes	48 hours
1 ½ hours	72 hours

5. At the end of the periods given in Table XIV transfer a loopful of the suspension of each culture to a tube of sterile broth. Incubate the cultures 48 hours at 37° C.

6. Examine the tubes for growth, comparing them with sterile tubes if necessary.

7. Construct a table on the regular note paper showing the results of the tests of both disinfectants, Table XV. Use a plus (+) sign when the organism is killed and a minus (-) sign when it is not killed.

TABLE XV.—Test of Disinfectants

Disinfectant	<i>Escherichia coli</i>				<i>Bacillus Anthracis</i>			
	Exposure time in minutes				Exposure time in hours			
	3	10	30	90	1.5	24	48	72

8.
- (a)

What is meant by sepsis?
- (b)

What is meant by antisepsis?
- (c)

What is meant by antiseptic?
- (d)

What is a disinfectant?
- (e)

What is a germicide?
- (f)

Are all antiseptics disinfectants?
- (g)

Name two gaseous disinfectants.
- (h)

What are the advantages of gaseous disinfectants?
- (i)

Give the advantages and disadvantages of the above disinfectants for stable disinfection.
- (j)

Give the best method of procedure in disinfecting a stable.
- (k)

What is meant by “terminal” disinfection? “concurrent” disinfection?

APPENDIX A.

OUTLINE FOR STUDY OF BACTERIAL CHARACTERS

A. PHYSIOLOGY (biochemical properties)

1. Relation to oxygen

Notice the distribution of the growth in the fermentation tubes and in tubes of broth and gelatin.

2. Pigment production.

Notice the growth on solid media.

3. Test reaction of all sugar broths, glycerin and plain broth by removing a drop with a sterile loop and placing it on litmus paper.

4. Note gas formation in any sugar broths and in glycerin broth. If gas is formed in fermentation tubes, measure it and determine the $H:CO_2$ ratio as shown by the instructor.

5. Observe if nitrates are reduced and if gas is formed (See Exercise 17.)

6. Test the starch agar culture for diastasic action if there is growth.

7. Habitat. Where the organism is found.

FIG. 22—NONLIQUEFYING GELATIN STAB CULTURES

1, *filiform*; 2, *beaded*; 3, *echinulate*; 4, *villous*; 5, *aborescent*

FIG. 23—LIQUEFYING GELATIN STAB CULTURES

6, *crateriform*; 7, *napiform*; 8, *infundibuliform*; 9, *saccate*; 10, *stratiform*

B. CULTURAL CHARACTERS

1. Gelatin Stab. Draw the culture. (See Figs. 22 and 23.)

I. Nonliquefying.

(a) Line of puncture: *filiform*, uniform needle-shaped growth; *beaded*, succession of small, disjointed colonies; *echinulate*, Prickly; *villous*, beset with unbranched hair-like extensions; *arborescent*, beset with root-like extensions.

(b) Surface growth. Same as for colonies on plate cultures.

II. Liquefying

(a) Type of liquefaction: *crateriform*, saucer-shaped; *napiform*, turnip-shaped; *infundibuliform*, funnel-shaped; *saccate*, sack-shaped; *stratiform*, the liquefaction descending in a horizontal plane.

(b) Character of the fluid: *clear*, *cloudy*, *flocculent*, *granular*.

2. Streak cultures (agar or potato). Draw the agar culture.

(a) Growth: *invisible*, *scanty*, *moderate*, *abundant*.

(b) Form: *filiform*, a narrow line; *echinulate*, growth along line of inoculation with toothed or pointed margins; *beaded*, a succession of small, disjointed colonies; *effused*, spreading; *villous*, *plumose*, *arborescent*. See Fig. 24.)

(c) Luster: *glistening*, *dull*, *cretaeous*.

(d) Optical characters: *opaque*, *translucent*, *opalescent*, *iridescent*.

(e) Elevation of growth: same as for plate cultures.

(f) Topography.

FIG. 24—AGAR STREAK CULTURES

1, *filiform*; 2, *echinulate*; 3, *beaded*; 4, *effused*; 5, *villous*

(g) Consistency: *slimy*, *butyrous*, of a consistency like butter; *viscid*, growth follows the needle when touched and withdrawn; *coriaceous*, growth tough, leathery; *brittle*, growth dry, friable under the needle.

(h) Medium discolored.

3. Beef-broth Cultures

(a) Growth or no growth.

(b) Condition of fluid: *clear*, *cloudy*.

(c) Surface membrane: *when formed*, color, consistency.

(d) Sediment: *amount*, *compact*, *flocculent*, *granular*, *viscid*.

4. Milk Cultures. Draw the litmus milk culture.

I. Curd formed.

- (a) Time required to curdle.
- (b) Character of curd: *hard or soft, solid or porous, changed or not, when boiled.*
- (c) Whey: *amount, clear or turbid.*
- (d) Reaction to litmus.
- (e) Digestion of curd (examine plain milk culture): *time required, reaction to litmus, solution cloudy or clear.*
- (f) Gas bubbles.
- (g) Odor.

II. Digestion without formation of curd.

III. No visible change even after boiling.

5. Plate Cultures.

- I. Surface colonies. Naked eye appearance. (See Fig. 25.) Draw a colony.

FIG. 25—NAKED EYE APPEARANCE OF COLONIES

A, punctiform; B, circular; C, oval; D, spindle-shaped; E, conglomerate; F, ameboid; G, rhizoid; H, curled; I, myceloid; J, filamentous.

- (a) *punctiform*, too small to be defined by the naked eye; *circular*: *oval; spindle-shaped; conglomerate*; an aggregate of similar

colonies; *ameboid*, very irregular; *rhizoid*, branched, root-like structure; *curled*, filaments in strands like curly hair; *myceloid*, *filamentous*, with the character of a mold.

(b) Size, approximate, expressed in millimeters.

FIG. 26—SURFACE ELEVATION OF COLONIES
a, flat; b, spreading; c, raised; d, convex; e, capitate; f, umbilicate; g, umbonate

- (c) Surface elevation (See Fig. 26): *flat*; *spreading*; *raised*; *convex*; surface a segment of a circle; *capitate*; surface a hemisphere *umbilicate*, depressed in the center; *umbonate*, elevated at the center.
- (d) Topography of surface: *smooth*; *contoured*, smoothly undulating, like the surface of a relief map; *rugose*, short, irregular folds due to shrinkage; *verrucose*, growth wartlike, with wart-like prominence.

II. Microscopic appearance. Draw a section and edge of the colony.

FIG. 27—INTERNAL STRUCTURE OF COLONIES
1, amorphous; 2, finely granular; 3, coarsely granular; 4, grumose; 5, gyrose; 6, reticulate; 7, filamentous

1, amorphous, 2, finely granular; 3, coarsely granular; 4, grumose; 5, gyrose; 6, reticulate; 7, filamentous

- (a) Internal structure (See Fig. 27) *amorphous*, no definite structure; *finely granular*, *coarsely granular*; *grumose*, appears clotted; *gyrose*, showing chinks or cracks; *reticulate*, netted; *filamentous*, tangled wavy threads.

FIG. 28—EDGES OF COLONIES
8, entire 9, undulate; 10, repand; 11, auriculate; 12, lacerate; 13, fimbriate; 14, ciliate

8, *entire*; 9, *undulate*; 10, *repand*; 11, *auriculate*; 12, *lacerate*; 13, *fimbriate*
14, *ciliate*

- (b) Edge of colony (See Fig. 28): *entire*; *undulate*, wavy; *repand*; *auriculate*; *lacerate*, irregularly cleft; *fimbriate*, fringed; *ciliate*, finely toothed.

III. Deep colonies. *Draw a colony.*

- (a) Color.
(b) Shape: *punctiform*, *lanceolate*, *oval*, *circular*, *spindle-shaped*, *irregular*, *branched*, *filamentous*.
(c) Translucency.

C. MORPHOLOGY

1. Form and arrangement (examine stained organisms on slides); *coccus*, single, and grouped; *diplococcus*; *streptococcus*; *rods*, single and in chains; *spirals*. (See Fig. 5).

2. Size. Measurements in terms of the micron.

3. Reaction to stains:

- (a) Simple stains; stains easily or with difficulty.
(b) Differential stains: Gram's stain.

4. Spores: Time required for formation, medium, position in cell.

5. Special characters.

- (a) Flagella. (Motility in hanging drop.)
(b) Capsule.
(c) Granules.
(d) Involution forms.

APPENDIX B.

GLOSSARY OF TERMS

Agar-hanging block, a small block of nutrient agar cut from the poured plate and placed on a cover glass, the surface next the glass having first been touched with a loop from a young fluid culture or with a dilution from the same. It is examined upside down, the same as a hanging drop.

Ameboid, assuming various shapes, like an ameba.

Amorphous, without visible differentiation in structure.

Aborescent, a branched, tree-like growth.

Beaded, in stab, or stroke, disjointed or semi-confluent colonies along the line of inoculation.

Brief, a few days, a week.

Brittle, growth dry, friable under the platinum needle.

Bullate, growth rising in convex prominences, like a blistered surface.

Butyrous, growth of a butter-like consistency.

Chains, short chains, composed of 2 to 8 elements; long chains, composed of more than 8 elements.

Ciliate, having fine, hair-like extensions, like cilia.

Cloudy, said of fluid cultures which do not contain pseudozoogloae.

Coagulation, the separation of casein from whey in milk. This may take place quickly or slowly, and as a result of either the formation of an acid or of a rennet-like ferment.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Convex, surface the segment of a circle, but flattened.

Coprophil, dung bacteria.

Coriaceous, growth tough, leathery, not yielding to the platinum needle.

Crateriform, round, depressed, due to the liquefaction of the medium.

Cretaceous, growth opaque and white, chalky.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.

Diastasic action, conversion of starch into water soluble substances by diastase.

Echinulate, in agar stroke, a growth along line of inoculation, with toothed or pointed margins; in stab cultures, growth beset with pointed outgrowths.

Effuse, growth thin, veily, unusually spreading.

Entire, smooth, having a margin destitute of teeth or notches.

Erose, border irregularly toothed.

Filamentous, growth composed of long, irregularly-placed or interwoven filaments.

Filiform, in stroke or stab cultures a uniform growth along line of inoculation.

Fimbriate, border fringed with slender processes, larger than filaments.

Floccose, growth composed of short curved chains, variously oriented.

Flocculent, said of fluids which contain pseudozooglae, that is, small adherent masses of bacteria of various shapes and floating in the culture fluid.

Fluorescent, having one color by transmitted light and another by reflected light.

Gram's stain, a method of differential bleaching after crystal violet, methyl violet, etc. The +mark is to be given only when the bacteria are deep blue or remain blue after counterstaining with a counterstain

Grumose, clotted.

Infundibuliform, form of a funnel or inverted cone.

Iridescent, like mother of pearl. The effect of very thin films.

Lacerate, having the margin cut into irregular segments, as if torn.

Lobate, border deeply undulate, producing lobes (see Undulate).

Long, many weeks or months.

Maximum temperature, temperature above which growth does not take place.

Medium, several weeks.

Membranous, growth thin, coherent, like membrane.

Minimum temperature, temperature below which growth does not take place.

Myceloid, colonies having the radiately filamentous appearance of mold colonies.

Napiform, liquefaction with the form of a turnip.

Nitrogen requirments, the necessary nitrogenous food. This is determined by adding to **nitrogen-free** media the nitrogen compound to be tested.

Opalescent, resembling the color of an opal.

Optimum temperature, temperature at which growth is most rapid.

Pellicle, in fluid, bacterial growth either forming a continuous or an interrupted sheet over the fluid.

Peptonized, said of curds dissolved by trypsin.

Persistent, many weeks or months.

Plumose, a fleecy or feathery growth.

Pseudozoogloae, clumps of bacteria, not separating readily in water, arising from imperfect separation or more or less fusion of the components, but not having the degree of compactness and gelatinization seen in zoogloae.

Pulvinate, in the form of a cushion, decidedly convex.

Punctiform, very minute colonies, at the limit of natural vision.

Raised, growth thick, with abrupt or terraced edges.

Rapid, developing in twenty-four to forty-eight hours.

Repand, wrinkled.

Rhizoid, growth of an irregular branched, or root-like character, as in *B. mycoides*.

Ring, (same as **rim**), growth at the upper margin of a liquid culture, adhering more or less closely to the glass.

Saccate, liquefaction the shape of an elongated sack, tubular, cylindrical.

Scum, floating islands of bacteria, an interrupted pellicle or bacterial membrane.

Short, applied to time, a few days, a week.

Slow, requiring five or six days or more for development.

Sporangia, cells containing endospores.

Spreading, growth extending much beyond the line of inoculation; that is, several millimeters or more.

Stratiform, liquefying to the walls of the tube at the top and then proceeding downward horizontally.

Thermal death point, the degree of heat required to kill young fluid cultures of an organism exposed for ten minutes (in thin-walled test tubes of a diameter not exceeding 20 mm.) in the thermal water bath. The water must be kept agitated, so that the temperature shall be uniform during the exposure.

Transient, a few days.

Turbid, cloudy, with flocculent particles; cloudy plus flocculence.

Umbonate, having a button-like raised center.

Undulate, border wavy, with shallow sinuses.

Vermiform contoured, growth like a mass of worms or intestinal coils.

Verrucose, growth wartlike, with wartlike prominences.

Villous, growth beset with hairlike extensions.

Viscid, growth follows the needle when touched and withdrawn: sediment on shaking, rises as a coherent swirl.

Zoogloaeae, firm gelatinous masses of bacteria, one of the most typical examples of which is the *Streptococcus mesenteroides* of sugar vats (*Leuconostoc mesenteroides*), the bacterial chains being surrounded by an enormously thickened firm covering, inside of which there may be one or many groups of the bacteria.

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