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A LABORATORY GUIDE
IN
SOIL BACTERIOLOGY

BY

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PREFACE.

Bacteriology has contributed, within the past few years, some important facts to our rapidly expanding knowledge of soils. It has shed a new light on the transformation and accumulation of nitrogen compounds in tilled land, as well as in uncultivated forest and prairie soils. It has taught us to understand more clearly the decomposition of cellulose and allied compounds, and the conservation of the potential energy of hydrogen and of methane. It has given the student of soils and of soil fertility a number of new methods for measuring differences in the quality and quantity of plant-food. It bids fair to supply us with still other methods that would be more adequate than any chemical methods yet devised, for the investigation of fundamental problems of soil fertility.

In view of the past achievements of soil bacteriology and of the promise of even greater usefulness in the future, no apology is needed for the publication of this little volume. The authors have felt, for some time, that there is room in every well-arranged course in soils for a series of exercises in soil bacteriology, and have, therefore, made the attempt to provide them. It is their hope that after preliminary instruction in general bacteriology and laboratory technique the "Guide" will prove useful to students of soils, and that time and service will permit the elimination of the imperfections and inadequacies that undoubtedly exist in it. Toward this end, friendly criticism will be appreciated. Meanwhile, the authors wish to express their indebtedness to Dr. R. E. Buchanan, of the Iowa State College, for many helpful suggestions.

A SHORT REFERENCE LIST OF BOOKS AND PERIODICALS FOR STUDENTS OF SOIL BACTERIOLOGY.

- Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, II. Abteilung.
Experiment Station Record.
Jahresbericht der Agriculturchemie.
Conn, Agricultural Bacteriology.
Kramer, Die Bakteriologie in ihren Beziehungen zur Landwirtschaft.
Kayser, Microbiologie Agricole.
Lafar, Handbuch der technischen Mycologie.
Lipman, Bacteria in Relation to Country Life.
Löhnis, Handbuch der landwirtschaftlichen Bakteriologie.
Meyer, Practicum der botanischen Bakterienkunde.
Stockhausen, Oekologie, Anhäufungen nach Beijerinck.
Voorhees and Lipman, Review of Investigations in Soil Bacteriology.
Wollny, Die Zersetzung der organischen Stoffe.
Annual Reports of the New Jersey, North Carolina and Wisconsin Experiment Stations.

APPARATUS NECESSARY FOR STUDENT USE.

1 Spatula.	8 Pipettes, 1 cc.
1 Staining forceps.	2 Platinum needles.
1 Forceps.	Lens paper.
1 Hollow slide.	24 Tumblers.
1 box Slides.	6 Beakers.
1 oz. Cover glasses.	1 Pipette box.
4 Wire baskets.	6 Copper flasks.
1 Cooking vessel.	4 Funnels.
1 Slide box.	6 Dropping bottles.
1 book Labels.	24 Flasks, Erlenmeyers, 250 cc.
20 Petri dishes.	6 Flasks, Erlenmeyers, 500 cc.
125 Test-tubes.	12 Flasks, Florence, 500 cc.
1 Pencil (for glass).	1 Flask, Florence, 1,000 cc.
12 Fermentation tubes.	Test-tube cleaner.
6 Pipettes, 10 cc.	Towel.
2 Pipettes, 5 cc.	Matches.
1 Pipette, 20 cc.	

GENERAL APPARATUS.

Jeffers counting plates.	Bunsen burners.
Hand lenses.	Large pans.
Graduated cylinders, 500 cc.	Ring stands.
Graduated cylinders, 100 cc.	Filter racks.
Thermometers.	Copper wire.
Colorimeters.	Distilling bulbs.
Rubber tubing.	Graduated Pipettes.
Pinch cocks.	Volumetric flasks, 100 cc.
Wire gauze.	Volumetric flasks, 500 cc.
Glass tubing.	Shears.
Stirring rods.	Burettes.
Porcelain cups.	Bottles.
Cork borers.	Pliers.
Files.	Gooch crucibles.
Mortars.	Tripods.
Funnels.	Microscopes and accessories.
Filter paper.	Balances and weights.
Plugging cotton.	Large Petri dishes.
Absorbent cotton.	

CHEMICALS REQUIRED.

Beef extract.	Maltose.	Immersion oil.
Salt.	Alcohol.	Stains.
Peptone.	Urea.	Sapolio.
Agar powder.	HgCl ₂ .	Soap.
Gelatin.	MgO (heavy).	Dried blood.
NaOH.	Paraffin.	Cottonseed meal.
K ₂ HPO ₄ .	Lead acetate.	Potatoes.
MgSO ₄ .	HCl.	Milk.
CaCl ₂ .	NH ₄ OH.	Phenolphthalein.
Fe ₂ Cl ₆ .	Hg.	Cochineal.
Mannite.	H ₂ SO ₄ .	Saccharose.
KNO ₃ .	K ₂ S.	Gypsum.
Citric acid.	Zinc dust.	MgCO ₃ .
Dextrose.	Sulfanilic acid.	MnSO ₄ .
KH ₂ PO ₄ .	Naphthylamine.	Sodium silicate.
(NH ₄) ₂ SO ₄ .	Acetic acid.	Sodium lactate.
Fe ₂ (SO ₄) ₃ .	Phenol.	Asparagin.
CaCO ₃ .	Diphenylamine.	Litmus.
NaNO ₂ .	Glycerine.	(NH ₄) ₂ C ₂ O ₄ .
Na ₂ CO ₃ .	Sodium acetate.	HNO ₃ .
NaNO ₃ .	BaCl ₂ .	
(NH ₄) ₃ PO ₄ .	Canada Balsam.	

EXERCISE I.

EXAMINATION OF SOIL INFUSIONS.

1. Prepare infusions of a sandy soil, a rich loam soil, and a sample of fresh manure by shaking 10 grams in 20 cc. of sterile water for 5 minutes.

2. Make hanging-drop and stained preparations from each of these infusions and examine under the microscope.

3. Describe the different organisms present and determine approximately the relative numbers of each.

4. Compare the number and character of the organisms in the different types of soil and in the manure.

EXERCISE II.

QUANTITATIVE EXAMINATION OF SOILS.

1. Fresh samples of soil taken with all precautions to avoid contamination are to be used.

2. Bring the bacteria into suspension by shaking 100 grams of each of the soils to be tested in 200 cc. of sterile water for 5 minutes.

3. Then, by means of sterile pipettes for each transfer, make dilutions of the various infusions thus:

- 1 cc. of the infusion into 99 cc. sterile water (a).
- 10 cc. of (a) into 90 cc. sterile water (b).
- 10 cc. of (b) into 90 cc. sterile water (c).
- 10 cc. of (c) into 90 cc. sterile water (d).

4. Transfer three 1 cc. portions of each (b), (c) and (d) dilution to sterile Petri dishes.

5. To each of one set of plates add 10 cc. of bouillon agar, liquefied, and cooled to 40° C.; to the second set, add 10 cc. of synthetic agar similarly liquefied and cooled; and to the third set, add 10 cc. of gelatin, liquefied and cooled to 30° C.

6. Mix thoroughly by tipping the plates back and forth and spread evenly over the dishes.

7. Incubate for 3-4 days at 20° C.

8. Count by means of a Jeffer's plate with the aid of a hand lens.

9. From the figures obtained calculate the number of bacteria per gram of soil.

EXERCISE III.

ISOLATION AND IDENTIFICATION OF SOME SOIL ORGANISMS
GROWN ON MEDIA EMPLOYED IN PREVIOUS EXERCISE.

1. By means of a platinum needle inoculate tubes of sterile bouillon from several well-isolated colonies, appearing on the bouillon agar, synthetic agar and gelatin plates made in the previous exercise; selecting those colonies most dissimilar in appearance.

2. Incubate 3-4 days at 20° C.

3. Plate out these cultures by the usual dilution method, using bouillon agar.

4. When pure cultures are obtained describe and identify the organisms by the usual morphological and cultural examination.

TRANSFORMATION OF NITROGEN COMPOUNDS. GROUP I. AMMONIFICATION.

Exercises IV. to XVII.

EXERCISE IV.

AMMONIFICATION IN PEPTONE SOLUTIONS.

1. Make up a 1% solution of Witte's peptone in water.
2. Measure out six 100 cc. portions into 500 cc. Jena flasks.
3. Plug and sterilize in the autoclave at 15 lbs. pressure for 10 minutes.
4. Prepare a soil infusion of each of the three soils to be examined, by shaking 100 grams in 200 cc. of sterile water for 5 minutes.
5. Inoculate the peptone solutions in duplicate with 20 cc. (= 10 grams soil) of the various infusions, transferring by means of sterile pipettes.
6. Incubate for 3 days at 20° C.
7. At the end of that time, remove the plugs, add about 100 cc. of water, 9-10 grams of heavy magnesium oxid, and a small piece of paraffin.
8. Distil off the ammonia present, collecting it in standard HCl and titrating against standard NH_4OH in the usual way.

EXERCISE V.

AMMONIFICATION IN SOILS.

1. Weigh off in tumblers six 100 gram portions of air-dry, sieved samples of the soil to be tested.

2. Arrange thus:

1 & 2—nothing.

3 & 4—5 grams of dried blood.

5 & 6—5 grams of cottonseed meal.

3. Stir in these materials very thoroughly by means of a sterile spatula.

4. Prepare an infusion of the soil by shaking 100 grams of a fresh sample with 200 cc. of sterile water for 5 minutes.

5. By means of sterile pipettes inoculate each of the six portions of soil with 10 cc. of this infusion.

6. Add sterile water to bring the moisture content up to the optimum for the soil, using 12 cc. additional for those portions containing the dried blood and cottonseed meal.

7. Cover with Petri dish covers and incubate for 7–8 days at 20° C.

8. Then transfer the soil to copper flasks, using about 250 cc. of water. Add 10–15 grams of MgO and a piece of paraffin and distil off the ammonia present, collecting it in standard acid and titrating against standard alkali.

EXERCISE VI.

THE INFLUENCE OF MOISTURE ON THE AMMONIFICATION OF
DRIED BLOOD.

1. Weigh off fourteen 100 gram portions of air-dry, sieved soil, in tumblers.
2. Add 5 grams of dried blood to each.
3. Stir thoroughly by means of a sterile spatula.
4. Add sterile water to the soil portions as follows:

1 & 2.....	2 cc.
3 & 4.....	4 cc.
5 & 6.....	8 cc.
7 & 8.....	12 cc.
9 & 10.....	18 cc.
11 & 12.....	24 cc.
13 & 14.....	30 cc.

5. Cover and incubate 7-8 days at 20° C.
6. Determine the ammonia formed by transferring to copper flasks, distilling and titrating in the usual way.

EXERCISE VII.

THE INFLUENCE OF AIR-DRYING ON THE AMMONIFYING POWER OF SOIL.

1. Divide some fresh, fertile soil into two portions. Place one portion in an air-tight receptacle and keep it in a cool place. Spread out the other portion, in a thin layer, in a warm room to facilitate air-drying. Weigh the air-dry soil, calculate the amount of moisture lost and restore it by the addition of sterile water.

2. Weigh off two 100 gram portions each of the fresh soil, and of the air-dry soil that has been restored to its original water content.

3. Add to each soil portion 5 grams of dried blood and stir with a sterile spatula.

4. If necessary increase water content to optimum by the addition of sterile water.

5. Cover, and incubate for 7-8 days at 20° C.

6. Determine the amount of ammonia formed by distilling and titrating in the usual way.

EXERCISE VIII.

THE INFLUENCE OF LIME AND MAGNESIA ON THE AMMONIFICATION OF DRIED BLOOD AND COTTONSEED MEAL.

1. Weigh off twelve 100 gram portions of air-dry, sieved soil, in tumblers.

2. Arrange thus:

- 1 & 2—5 grams dried blood.
- 3 & 4—5 grams dried blood + 1 gram CaCO_3 .
- 5 & 6—5 grams dried blood + 1 gram MgCO_3 .
- 7 & 8—5 grams cottonseed meal.
- 9 & 10—5 grams cottonseed meal + 1 gram CaCO_3 .
- 11 & 12—5 grams cottonseed meal + 1 gram MgCO_3 .

3. Stir thoroughly with a sterile spatula.

4. To each add 10 cc. of a soil infusion prepared by shaking 100 grams of a fresh sample of soil in 200 cc. of sterile water for 5 minutes.

5. Add sterile water to bring the moisture content up to the optimum for the soil and then 12 cc. additional for the nitrogenous materials.

6. Cover and incubate 7–8 days at 20°C .

7. Then determine the ammonia formed by transferring and distilling in the usual way.

EXERCISE IX.

THE INFLUENCE OF FINENESS OF DIVISION OF DRIED BLOOD
ON ITS AMMONIFICATION.

1. Weigh off in tumblers eight 100 gram portions of air-dry, sieved samples of soil.

2. Arrange thus:

1 & 2—5 grams dried blood, unsieved.

3 & 4—5 grams dried blood through 2 mm. sieve.

5 & 6—5 grams dried blood through 1 mm. sieve.

7 & 8—5 grams dried blood through $\frac{1}{2}$ mm. sieve.

3. Stir all very thoroughly by means of a sterile spatula.

4. Prepare a soil infusion of the soil employed by shaking 100 grams of a fresh sample in 200 cc. of sterile water for 5 minutes.

5. By means of sterile pipettes inoculate each of the eight portions of soil with 10 cc. of this infusion.

6. Add sterile water to bring the moisture content up to the optimum for the soil, using 12 cc. additional for the dried blood.

7. Cover with Petri dish covers and incubate 7–8 days at 20° C.

8. Then transfer the soil to copper flasks and determine the ammonia present as described in Exercise V.

EXERCISE X.

THE INFLUENCE OF DEXTROSE ON THE AMMONIFICATION OF
DRIED BLOOD AND COTTONSEED MEAL.

1. Weigh off twelve 100 gram portions of air-dry, sieved soil, in tumblers.

2. Arrange thus:

1 & 2—5 grams dried blood.

3 & 4—5 grams dried blood + 1 gram dextrose.

5 & 6—5 grams dried blood + 3 grams dextrose.

7 & 8—5 grams cottonseed meal.

9 & 10—5 grams cottonseed meal + 1 gram dextrose.

11 & 12—5 grams cottonseed meal + 3 grams dextrose.

3. Stir thoroughly with a sterile spatula.

4. To each add 10 cc. of a soil infusion prepared by shaking 100 grams of a fresh sample of soil in 200 cc. of sterile water for 5 minutes.

5. Add sterile water to bring the moisture content up to the optimum for the soil and then 12 cc. additional for the nitrogenous materials.

6. Cover and incubate 7–8 days at 20° C.

7. Then determine the ammonia formed by transferring and distilling in the usual way.

EXERCISE XI.

AMMONIFICATION AS AFFECTED BY DIFFERENCES IN THE MECHANICAL AND CHEMICAL COMPOSITION OF SOILS.

1. Weigh off two 100 gram portions each of air-dry sandy, clay, loam and peaty soil, in tumblers.
2. Add 5 grams of dried blood to each and stir by means of a sterile spatula.
3. To each add 10 cc. of a soil infusion prepared by shaking 100 grams of a fresh sample of soil in 200 cc. of sterile water for 5 minutes.
4. Cover and incubate for 7-8 days at 20° C.
5. Then determine the ammonia formed by transferring and distilling in the usual way.

EXERCISE XII.

THE AMMONIFICATION OF DRIED BLOOD BY PURE CULTURES
OF SOIL ORGANISMS.

1. Weigh off twelve 100 gram portions of sieved, air-dry soil; add to each 5 grams of dried blood; stir thoroughly.

2. Transfer to Erlenmyer flasks; increase water to optimum content, using 12 cc. additional for the dried blood; plug; sterilize in autoclave; cool.

3. Inoculate as follows, using 1 cc. of vigorous cultures 2-3 days old:

1 & 2 *B. mycoides*.

3 & 4—*B. subtilis*.

5 & 6—*B. coli*.

7 & 8—*B. pinatus* or some other member of the coli group.

9 & 10—*B. proteus*.

11 & 12—Another member of the proteus group.

4. Incubate for 7-8 days at 20° C.

5. Transfer to copper flasks, distil, and determine the ammonia formed, in the usual way.

EXERCISE XIII.

THE AVAILABILITY OF NITROGENOUS ORGANIC MATTER AS INDICATED BY ITS AMMONIFICATION.

1. Weigh off twelve 100 gram portions of air-dry, sieved soil, in tumblers.

2. Make additions of organic nitrogen, using amounts equivalent to 155 milligrams of nitrogen, as follows:

3.

1 & 2—concentrated tankage.

3 & 4—dried blood.

5 & 6—cottonseed meal.

7 & 8—bone meal.

9 & 10—horse manure (dried and ground).

11 & 12—cow manure (dried and ground).

4. Stir in these materials thoroughly by means of a sterile spatula.

5. Add sterile water to bring the moisture content of the soil up to the optimum, making further additions in each case to provide organic matter containing 70% of water.

6. Cover with Petri dish covers and incubate for 7–8 days at 20° C.

7. Transfer to copper flasks, distil and determine the ammonia formed, in the usual way.

EXERCISE XIV.

THE RELATIVE AMOUNTS OF AMMONIA AND CARBON DIOXID
FORMED IN THE AMMONIFICATION OF NITROGENOUS
MATERIALS.

1. Weigh off six 100 gram portions of air-dry, sieved soil, in tumblers.

2. Add dried blood and cottonseed meal as follows:

1 & 2—nothing.

3 & 4—5 grams dried blood.

5 & 6—5 grams cottonseed meal.

3. Stir thoroughly. Add optimum proportion of water and 12 cc. additional for the dried blood and cottonseed meal.

4. Cover tumblers and place them under bell-jars of 4-5 liters capacity, standing on vaselined ground glass plates. At the same time place under each bell-jar an Erlenmeyer flask containing 10 cc. of a standard barium hydrate solution each cc. of which is equivalent to approximately 3 milligrams of carbon dioxide.

5. Remove and titrate the barium hydrate at the end of each day for six consecutive days, replacing it by fresh portions. The air under each bell-jar is renewed at the same time, by lifting it from the plate and turning it up and down several times. Record the amount of carbon dioxide produced.

6. At the end of six days transfer the contents of each tumbler into a copper flask, add water and magnesium oxide, distil and titrate.

7. Two additional bell-jars without soil may be added to the series to show the amount of carbon dioxid furnished by the air. This amount is to be subtracted from that found in each of the other bell-jars.

8. From the amounts of carbon dioxid and ammonia found calculate the relative oxidation of the carbon and nitrogen in the materials.

9. If desired, corresponding soil portions, kept under the same conditions outside of bell-jars, may be used for determining the *daily* production of ammonia. This would require ten additional portions with dried blood and ten additional portions with cottonseed meal.

EXERCISE XV.

THE INFLUENCE OF DEXTROSE ON THE FORMATION OF CARBON DIOXID AND AMMONIA FROM DRIED BLOOD.

1. Weigh off eight 100 gram portions of air-dry, sieved soil, in tumblers.

2. Arrange series under bell-jars as in preceding exercises as follows:

1 & 2—no soil.

3 & 4—100 grams of soil.

5 & 6—100 grams of soil + 5 grams dried blood.

7 & 8—100 grams of soil + 3 grams dextrose.

9 & 10—100 grams of soil + 5 grams dried blood +
3 grams dextrose.

3. Stir thoroughly; increase soil moisture to optimum by the addition of sterile water; use 12 cc. additional for the dried blood and 7 cc. for the dextrose.

4. Titrate daily the barium hydrate solutions and replace by fresh portions for six consecutive days.

5. At the end of six days transfer the soil portions to copper flasks; add water and magnesium oxid; distil and titrate.

6. Calculate rate of decomposition of the dried blood, as influenced by the dextrose.

EXERCISE XVI.

AMMONIFICATION OF UREA.

1. Measure off six 100 gram portions of "urea solution" into 500 cc. flasks.

2. Plug and sterilize in the autoclave at 10 lbs. pressure for 10 minutes.

3. Arrange thus:

1 & 2—nothing.

3 & 4—Add 20 cc. (= 10 gms. soil) of a 5-minute infusion of a fresh garden soil.

5 & 6—Add 20 cc. (=10 gms. soil) of a 5-minute infusion of a pasteurized garden soil (to pasteurize the soil, heat it to 70° C. for 30-40 minutes).

4. Incubate 2-3 days at 20° C.

5. Pipette off two 10 cc. portions of each culture and titrate the ammonia present with $\frac{N}{20}$ HCl, using cochineal as an indicator.

6. From the results obtained calculate the total quantity of ammonia produced and the per cent. of urea transformed.

EXERCISE XVII.

ISOLATION OF UREA BACTERIA.

1. By means of a platinum loop make transfers from flasks 3, 4, 5 and 6 in the preceding experiment into tubes of sterile water.

2. From these dilutions inoculate tubes of sterile liquefied and cooled (40° C.) "urea agar" in series and plate in the usual way.

3. Incubate for 3-4 days at 20° C.

4. From several well-isolated colonies inoculate into tubes of "urea solution."

5. Incubate 3-4 days at 20° C.

6. Test for the production of ammonia by inserting strips of filter paper moistened with Nessler's solution in the upper part of each tube.

7. Examine the cultures in hanging drop and stained preparations.

8. Identify by the usual cultural and morphological examinations.

GROUP II. NITRIFICATION.**Exercises XVIII. to XXIV.****EXERCISE XVIII.****NITRATE FORMATION IN SOLUTIONS.**

1. Measure off twelve 100 cc. portions of "solution for nitrite formation" into 250 cc. Erlenmeyer flasks. Plug and sterilize as usual.

2. Inoculate in duplicate with 20 cc. portions (= 10 gms. soil) of 5-minute infusions of the two soils to be tested.

3. Incubate 7-8 days at 20° C.

4. Test qualitatively for the presence of nitrites by the Griess colorimetric method.

5. If nitrites are found, by means of a platinum loop, inoculate from the growth in each flask into other flasks of the same solution.

6. Incubate these new cultures 7-8 days.

7. Test again for the presence of nitrites, and if positive results are obtained repeat the inoculation, using still other flasks of solution.

8. After 7-8 days' incubation, filter and wash and determine the nitrites present by the Griess colorimetric method.

9. Calculate the per cent. of the original ammonia nitrogen changed to nitrite.

EXERCISE XIX.

NITRIFICATION IN SOLUTION.

1. Measure off four 100 cc. portions of "nitrite solution for nitrification" into 250 cc. Erlenmeyer flasks.

2. Plug and sterilize as usual.

3. Then inoculate in duplicate with 20 cc. portions (= 10 gms. soil) of 5-minute infusions of the two soils to be tested.

4. Incubate for 4 weeks at 20° C.

5. At the end of that time, filter and wash thoroughly and determine the nitrates present in some convenient aliquot of the solutions by means of the phenol-sulfonic acid method.

6. Calculate the per cent. of the original nitrite nitrogen nitrified by the soils.

Handwritten notes:
1. 100 cc. of 10% soil
2. 100 cc. of 10% soil
3. 100 cc. of 10% soil
4. 100 cc. of 10% soil
5. 100 cc. of 10% soil
6. 100 cc. of 10% soil
7. 100 cc. of 10% soil
8. 100 cc. of 10% soil
9. 100 cc. of 10% soil
10. 100 cc. of 10% soil
11. 100 cc. of 10% soil
12. 100 cc. of 10% soil
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44. 100 cc. of 10% soil
45. 100 cc. of 10% soil
46. 100 cc. of 10% soil
47. 100 cc. of 10% soil
48. 100 cc. of 10% soil
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92. 100 cc. of 10% soil
93. 100 cc. of 10% soil
94. 100 cc. of 10% soil
95. 100 cc. of 10% soil
96. 100 cc. of 10% soil
97. 100 cc. of 10% soil
98. 100 cc. of 10% soil
99. 100 cc. of 10% soil
100. 100 cc. of 10% soil

EXERCISE XX.

NITRIFICATION IN SOILS.

1. Weigh off in tumblers six 100 gram portions of an air-dry, sieved sample of the soil to be tested.

2. Arrange thus:

Bareh Soil

1 & 2—nothing.

3 & 4—100 mgs. $(\text{NH}_4)_2\text{SO}_4$.

5 & 6—200 mgs. dried blood.

3. Stir in these materials very thoroughly by means of a sterile spatula.

4. Add 10 cc. of a 5-minute infusion of a fresh sample of the soil to each.

5. Add sterile water to bring the moisture conditions up to the optimum for the soil.

6. Cover with Petri dish covers.

7. Weigh on a torsion balance and record the total weight on the tumblers.

8. Incubate for 4 weeks at 20°C .

9. Weigh every ten days and by means of sterile water restore that lost by evaporation.

10. At the end of the incubation period add water and about 2 gms. of lime, and shake thoroughly in a wide-mouth bottle.

11. Filter through folded filter paper and wash thoroughly.

12. Make up to some convenient volume and determine the nitrate present in aliquot portions, by the phenol-sulfonic acid method.

13. Calculate the total nitrate-nitrogen present in the 100 gram portions of soil.

From the data at hand and the known composition of the ammonium sulfate and the dried blood, calculate the proportion of nitrogen nitrified in each of these materials by the soil examined.

EXERCISE XXI.

THE RATE OF OXIDATION OF AMMONIUM SULFAT IN SOLUTIONS AND IN SOILS IN THE PRESENCE AND ABSENCE OF CaCO_3 .

1. Measure off in 250 cc. Erlenmeyer flasks eight 100 cc. portions of "solution for nitrite formation," adding the CaCO_3 required to only four of these portions.

2. Plug and sterilize in the usual way.

3. Weigh off eight 100 gram portions of air-dry, sieved soil, in tumblers.

4. Add 100 mgs. $(\text{NH}_4)_2\text{SO}_4$ to each.

5. Add 1 gm. of CaCO_3 to four of these.

6. Stir in thoroughly by means of a sterile spatula.

7. Arrange thus:

1, 2, 3 & 4—100 cc. solution.

5, 6, 7 & 8—100 cc. solution + 1 gm. CaCO_3 .

9, 10, 11 & 12—100 gms. soil + 100 mgs. $(\text{NH}_4)_2\text{SO}_4$.

13, 14, 15 & 16—100 gms. soil + 100 mgs. $(\text{NH}_4)_2\text{SO}_4$ + 1 gm. CaCO_3 .

8. Inoculate both soils and solutions with 10 cc. (= 5 gms. soil) of a 5-minute infusion of a fresh sample of the soil employed.

9. Add sterile water to the soil portions to bring the moisture conditions up to the optimum and cover the tumblers with Petri dish covers.

10. Place in the incubator at 20°C .

11. Weigh every ten days and with sterile water replace that lost by evaporation.

12. At the end of two weeks determine, by the phenol-sulfonic acid method the nitrates present in samples 1, 2, 5, 6, 9, 10, 13 and 14; filtering the solutions and leaching the soil after additions of a little lime, making up to convenient volumes and aliquoting in the usual way.

13. At the end of four weeks the nitrates present in the remaining samples are determined similarly.

14. Calculate the per cent. of ammonia nitrogen nitrified in the soils and in the solutions, in the same length of time, in the presence or absence of lime, and compare the rate of oxidation under these conditions.

EXERCISE XXII.

THE INFLUENCE OF SOLUBLE ORGANIC MATTER ON NITRIFICATION IN SOILS.

1. Weigh off six 100 gram portions of air-dry, sieved soil, in tumblers.

2. Add 100 mgs. $(\text{NH}_4)_2\text{SO}_4$ to each.

3. Make further additions as follows:

1 & 2—nothing.

3 & 4—1 gram dextrose.

5 & 6—5 grams dextrose.

4. Stir thoroughly by means of a sterile spatula.

5. Inoculate all with 10 cc. (= 5 gms. soil) of a 5-minute infusion of a fresh sample of the soil.

6. Add sterile water to bring the moisture conditions up to the optimum for the soil.

7. Cover with Petri dish covers, weigh, and record the weight on the tumblers.

8. Incubate 4 weeks at 20° C.

9. Weigh every ten days and by means of sterile water restore that lost by evaporation.

10. At the end of the incubation period add water and about 2 gms. of lime; shake, filter, wash and determine the nitrates present in aliquot portions of the leachings as usual.

EXERCISE XXIII.

THE AVAILABILITY OF NITROGENOUS ORGANIC MATTER AS INDICATED BY ITS NITRIFICATION.

1. Weigh off sixteen 100 gm. portions of air-dry, sieved soil, in tumblers.

2. Make additions of the following materials in such amounts that the nitrogen present in all cases shall be the same as is supplied by 100 mgs. ammonium sulfate.

3. Arrange thus:

- 1 & 2—nothing.
- 3 & 4—100 mgs. ammonium sulfate.
- 5 & 6—dried blood.
- 7 & 8—concentrated tankage.
- 9 & 10—cottonseed meal.
- 11 & 12—bone meal.
- 13 & 14—horse manure (dried and ground).
- 15 & 16—cow manure (dried and ground).

4. Stir all thoroughly by means of a sterile spatula.

5. Add sterile water to bring the moisture conditions up to the optimum for the soil.

6. Cover with Petri dish covers; weigh and record the weight on the tumblers.

7. Incubate 4 weeks at 20° C.

8. Weigh every ten days and with sterile water replace that lost by evaporation.

9. At the end of the incubation period add water and a little lime; shake, filter, make up to some convenient volume and determine the nitrates present in aliquots, by the phenol-sulfonic acid method.

10. From the results obtained, subtracting in each case the amounts produced in the soil itself, calculate the per cent. of organic nitrogen transformed to nitrate.

11. Then taking the availability of $(\text{NH}_4)_2\text{SO}_4$ at 100 as a basis, calculate the relative availabilities of the other materials.

12. Compare the results obtained in this experiment with those secured in Exercise XIII., showing the comparative availability indicated by ammonification and nitrification tests.

EXERCISE XXIV.

THE ISOLATION OF NITRIFYING BACTERIA BY THE MAGNESIA-GYPSUM BLOCK METHOD.

1. Measure off four 100 cc. portions of "solution for nitrite formation" in 250 cc. Erlenmeyer flasks.
2. Plug and sterilize as usual.
3. Inoculate with 10 cc. (= 5 gm. soil) of a 5-minute infusion of a fresh sample of soil.
4. Incubate 7-8 days at 20° C.
5. Test qualitatively for the production of nitrites by the Griess colorimetric method.
6. If nitrites are present, by means of a platinum loop inoculate from these cultures on the surface of the magnesia-gypsum blocks, spreading the inoculum uniformly over the surface.
7. Incubate 10-14 days at 20° C.; at the end of six days replacing the liquid surrounding the gypsum block with a fresh supply.
8. Inoculate from some well isolated colonies to other gypsum blocks; incubate as usual and describe carefully the character of the growth.
9. Test qualitatively the solution surrounding the blocks for ammonia, nitrites and nitrates.
10. Examine the pure cultures obtained in hanging-drop and stained preparations and describe the character of the organisms.

GROUP III. DENITRIFICATION.**Exercises XXV. to XXIX.****EXERCISE XXV.****DENITRIFICATION IN SOLUTION.**

1. Place 10 cc. of the "Giltay" solution in each of six test tubes.

2. Plug and sterilize in the autoclave.

3. Arrange thus:

1 & 2—nothing.

3 & 4—0.1 gm. soil.

5 & 6—0.1 gm. horse manure.

4. Incubate 7–8 days at 20° C.

5. Test qualitatively for nitrates, using a diphenylamine solution in sulphuric acid—a blue coloration indicates the presence of nitrates.

EXERCISE XXVI.

ISOLATION OF DENITRIFIERS.

1. By means of a sterile platinum loop make transfers from tubes 3, 4, 5 and 6 of the previous experiment, into tubes of sterile water.

2. Then inoculate from these dilutions into three tubes of liquefied and cooled (40° C.) "Giltay" agar in series.

3. Pour Petri dishes as usual.

4. Incubate 4-5 days at 20° C.

5. Inoculate from several well isolated colonies appearing on these plates into tubes of sterile "Giltay" solution.

6. Incubate 4-5 days at 20° C., and if cultures are not pure replate as before until pure cultures are obtained.

7. Test qualitatively for the disappearance of nitrates as in the preceding experiment.

8. If nitrates are absent inoculations are made from each culture into 100 cc. portions of "Giltay" solution in 500 cc. Jena flasks, and when a qualitative test shows that all nitrates have disappeared the solutions are digested by the regular Kjeldahl method and the total nitrogen determined. The difference between the amount of nitrogen found and that supplied as nitrate will give the loss of nitrogen as gas.

9. Examine the pure cultures of denitrifiers obtained and identify by the usual cultural and morphological descriptions.

EXERCISE XXVII.

DENITRIFICATION BY PURE CULTURES OF DIFFERENT SPECIES.

1. Place 50 cc. of the "Giltay" solution in each of eight 250 cc. Erlenmeyer flasks.

2. Plug and sterilize in the autoclave.

3. Inoculate as follows:

1 & 2—sterile.

3 & 4—*Ps. pyocyaneus*.

5 & 6—*B. denitrificans*.

7 & 8—*B. coli*.

4. Incubate for 7–8 days at 20° C.

5. Divide each culture into three equal portions; determine:

(a) Total nitrogen by digestion, according to the modified Kjeldahl method.

(b) Ammonia nitrogen by distillation with magnesium oxide.

(c) Nitrate and ammonia nitrogen according to the Ulsch method.

The total less the ammonia and nitrate nitrogen equals the organic nitrogen. The nitrogen found by the Ulsch method less the ammonia nitrogen, equals the nitrate nitrogen.

EXERCISE XXVIII.

DENITRIFICATIONS IN SOILS.

1. Weigh off six 100 gram portions of air-dry, sieved soil, in tumblers.

2. Add 0.5 grams of NaNO_3 to each.

3. Arrange thus:

1 & 2—Infusion Soil 1.

3 & 4—Infusion Soil 2.

5 & 6—Infusion Soil 3.

4. Add 10 cc. (= 5 grams soil) of 5-minute infusions of fresh samples of the three soils to be tested.

5. Add sterile water to bring the moisture conditions up to the optimum.

6. Cover with Petri dish covers and incubate for 10–14 days at 20° C.

7. At the end of that time add water and a little lime; shake, filter, wash, make up to a convenient volume and determine the nitrates in aliquots of the leachings.

8. Dry the residue, grind, and analyze for total nitrogen by the regular Kjeldahl method.

9. Grind a sample of the air-dry soil used as the medium and determine the nitrogen present.

10. The difference between the total amount of nitrogen present at the beginning of the experiment (the nitrogen in the soil + the nitrate added) and at the end represents the amount which has been lost by denitrification, and the relative denitrifying powers of the soils may thus be determined.

EXERCISE XXIX.

CONDITIONS FAVORING DENITRIFICATION IN SOILS. EXCESSIVE MOISTURE AND EXCESSIVE ORGANIC MATTER.

1. Weigh off twelve 100 gram portions of air-dry, sieved soil, in tumblers.

2. Add 0.5 gram NaNO_3 to each.

3. Arrange thus:

1 & 2—nothing.

3 & 4—20 grams of horse manure.

5 & 6—20 grams of dextrose.

7 & 8—nothing.

9 & 10—20 grams of horse manure.

11 & 12—20 grams of dextrose.

4. Stir in these materials very thoroughly by means of a sterile spatula.

5. To each add 10 cc. of a soil infusion prepared by shaking 100 grams of a fresh soil in 200 cc. of sterile water for 5 minutes.

6. Add sterile water to soil portions 1-6 to bring the moisture content up to the optimum, or 50% saturation, making such further additions where the horse manure and dextrose are added that organic matter containing 70% water shall be present.

7. To soil portions 6-12 add sterile water to bring the moisture content up to 100% saturation, making further additions for the organic matter as before.

8. Incubate 10-14 days at 20° C.

9. Add water and a little lime; filter, wash, make up to some convenient volume and determine the nitrates present in aliquots.

10. Dry and grind the residues and determine the total nitrogen present by the regular Kjeldahl method.

11. From the results obtained calculate the per cent. of the NaNO_3 denitrified and the effect of excessive moisture or excessive organic matter on the loss of nitrogen.

GROUP IV. THE CHANGE OF SIMPLE INTO MORE COMPLEX COMPOUNDS.

Exercises XXX. to XXXI.

EXERCISE XXX.

THE TRANSFORMATION OF AMMONIA NITROGEN INTO PROTEIN.

1. Measure off eight 100 cc. portions of "Ammonia Nitrogen Transformation" solution into Erlenmeyer flasks.

2. Plug and sterilize in the autoclave.

3. Inoculate thus:

1 & 2—1 cc. vigorous culture of *B. vulgaris*.

3 & 4—1 cc. vigorous culture of *B. mirabilis*.

5 & 6—20 cc. (= 10 gm. soil) filtered, 5-minute infusion Soil 1.

7 & 8—20 cc. (= 10 gm. soil) filtered, 5-minute infusion Soil 2.

4. Soils 1 and 2 should be of different types.

5. Incubate 10–14 days at 20° C.

6. Then add basic lead acetate to precipitate the protein.

7. Filter, wash, and digest the residue by the regular Kjeldahl method for total N.

8. The results obtained show the power of the pure cultures and the soils to transform ammonia nitrogen into protein.

EXERCISE XXXI.

THE TRANSFORMATION OF NITRATE NITROGEN INTO PROTEIN.

1. Measure off eight 100 cc. portions of "nitrate-nitrogen transformation" solution into Erlenmeyer flasks.

2. Plug and sterilize in the autoclave.

3. Inoculate thus:

1 & 2—1 cc. vigorous culture *B. subtilis*.

3 & 4—1 cc. vigorous culture *B. mycoides*.

5 & 6—20 cc. (= 10 gm. soil) filtered, 5-minute infusion Soil 1.

7 & 8—20 cc. (= 10 gm. soil) filtered, 5-minute infusion Soil 2.

4. Incubate 10–14 days at 20° C.

5. Precipitate the protein present by means of basic lead acetate.

6. Filter, wash and digest the residue by the regular Kjeldahl method for total N.

7. The results obtained show the power of the pure cultures and the soils to transform nitrate nitrogen into protein.

GROUP V. NON-SYMBIOTIC NITROGEN FIXATION.

Exercises XXXII. to XLIII.

EXERCISE XXXII.

NITROGEN FIXATION BY SOILS IN MANNITE SOLUTIONS.

1. Measure off four 100 cc. portions of mannite solution and place in 500 cc. flasks.
2. Plug and sterilize in the autoclave as usual.
3. Inoculate duplicate portions with 20 cc. of a freshly prepared 5-minute infusion of two soils of different types.
4. Incubate for 10 days at 20° C.
5. Make transfers for the isolation of pure cultures in the succeeding experiment.
6. Remove plugs, add 0.7 gm. Hg. and 20 cc. of H_2SO_4 and determine the nitrogen present by the regular Kjeldahl method.

EXERCISE XXXIII.

THE ISOLATION AND DESCRIPTION OF AZOTOBACTER.

1. By means of a platinum loop make transfers from the cultures obtained in the preceding experiment into tubes of sterile mannite solution.

2. Incubate 6-7 days at 20° C.

3. Then inoculate from these cultures into tubes of sterile water.

4. From each of these dilutions make transfers into three tubes of sterile, liquefied, and cooled (to 40° C.) mannite agar, in series, and plate in the usual way.

5. Incubate 6-7 days at 20° C.

6. With a platinum needle, inoculate from several well isolated, dissimilar colonies into tubes of sterile mannite solution.

7. Incubate 6-7 days at 20° C.

8. Make microscopic stained and hanging-drop preparations from the pure cultures obtained and describe the characteristics of the organisms.

9. Inoculate from each culture into flasks containing 100 cc. of sterile mannite solution.

10. Incubate 6-7 days at 20° C.

11. Add Hg. and H₂SO₄ and digest, determining the nitrogen present by the regular Kjeldahl method.

12. Describe the growth of each of the organisms on agar plates and in mannite solutions, and identify them.

EXERCISE XXXIV.

NITROGEN FIXATION IN SOILS.

1. Weigh off six 100 gram portions of air-dry, sieved soil, in tumblers.
2. Add 1 gram of mannite to each.
3. Stir in thoroughly by means of a sterile spatula.
4. Arrange thus:

1 & 2—Infusion Soil 1.

3 & 4—Infusion Soil 2.

5 & 6—Infusion Soil 3.

5. Inoculate in duplicate with 10 cc. (= 5 gms. soil) 5-minute infusions of three soils of different types.

6. Add sterile water to bring the moisture conditions up to the optimum for the soil.

7. Cover with Petri dish cover and incubate 10–14 days at 20° C.

8. Remove, dry, grind and analyze for total nitrogen by the regular Kjeldahl method.

9. Grind a sample of the air-dry soil used as the medium and determine the total nitrogen present.

10. The difference between the total nitrogen in the beginning and at the end of the experiment gives the amount of nitrogen fixed in the soil, and the relative fixing powers of the different soils may thus be determined.

EXERCISE XXXV.

DESCRIPTION OF PURE CULTURES OF AZOTOBACTER.

1. Pure cultures of *Az. vinelandii*, *Az. beijerinckii* and *Az. chroococcum* are to be carefully examined and described for future reference.
2. Make microscopic stained and hanging drop preparations and note the characteristics of the various organisms.
3. Inoculate into tubes of mannite solution, incubate 4-5 days at 20° C. and describe the growth.
4. Prepare mannite agar plates in the usual way, incubate 4-5 days at 20° C. and study the colonies developed.
5. Inoculate on potatoes and after incubation describe the growth.
6. Make gelatin stabs, gelatin plates, sugar broth and milk cultures and describe the growth obtained.
7. Tabulate the results of the examinations.

EXERCISE XXXVI.

NITROGEN FIXATION BY PURE CULTURES OF AZOTOBACTER IN SOLUTIONS AND IN SOILS.

1. Measure off six 100 cc. portions of mannite solution into 500 cc. Jena flasks.
2. Plug and sterilize in the autoclave as usual.
3. Weigh off six 100 gram portions of air-dry, sieved soil, in tumblers.
4. Add 1 gram of mannite to each.
5. Stir thoroughly by means of a sterile spatula.
6. Inoculate with 1 cc. of vigorous cultures thus:

1 & 2—Mannite solution, <i>Az. beijerincki</i> .
3 & 4—Mannite solution, <i>Az. chroococcum</i> .
5 & 6—Mannite solution, <i>Az. vinelandii</i> .
7 & 8—Soil, <i>Az. beijerincki</i> .
9 & 10—Soil, <i>Az. chroococcum</i> .
11 & 12—Soil, <i>Az. vinelandii</i> .

7. Add sterile water to the soil portions to bring the moisture conditions up to the optimum for the soil.
8. Cover with Petri dish covers.
9. Incubate soils and solutions 7–8 days at 20° C.
10. Determine the nitrogen in the solutions by the regular Kjeldahl method.
11. Dry and grind the soil portions and determine the nitrogen present.
12. Grind a sample of the air-dry soil used as the medium and determine the nitrogen present.

13. The difference between the nitrogen present in the soil medium and in the cultures after incubation gives the amount of nitrogen fixed in the soil by the pure cultures.

14. Compare the amounts of nitrogen fixed by the pure cultures in solution with those fixed in the soil itself.

EXERCISE XXXVII.

THE INFLUENCE OF DIFFERENT CARBOHYDRATES ON NITROGEN
FIXATION BY AZOTOBACTER.

1. Make up one liter of "mannite" solution without adding the mannite.
2. Divide into ten 100 cc. portions in 500 cc. flasks.
3. Make the following additions.

1 &	2—1.5 gms. mannite.
3 &	4—1.5 gms. dextrose.
5 &	6—1.5 gms. glycerine.
7 &	8—1.5 gms. 1% ethyl alcohol.
9 &	10—1.5 gms. sodium acetate.

4. Plug and sterilize in the autoclave as usual.
5. Inoculate each with 1 cc. of a vigorous culture of *Az. vinelandii*.
6. Incubate 6–7 days at 20° C.
7. Add 0.7 gm. Hg. and 20 cc. H_2SO_4 and determine the nitrogen present by the regular Kjeldahl process.
8. The effect of different carbon sources on the amount of nitrogen fixed is thus determined.

EXERCISE XXXVIII.

THE EFFECT OF VARYING REACTION OF THE MEDIUM ON
NITROGEN FIXATION BY AZOTOBACTER.

1. Make up 1200 cc. of "mannite" solution as described except that it is not neutralized.
2. Divide into twelve 100 cc. portions in 300 cc. flasks.
3. Neutralize thus:

1 & 2— $\frac{1}{4}$ of the acidity neutralized	}	Az. vinelandii.
3 & 4— $\frac{1}{2}$ of the acidity neutralized		
5 & 6—all of the acidity neutralized		
7 & 8— $\frac{1}{4}$ of the acidity neutralized	}	Az. chroococcum.
9 & 10— $\frac{1}{2}$ of the acidity neutralized		
11 & 12—all of the acidity neutralized		

4. Plug and sterilize in the autoclave as usual.
5. Inoculate with 1 cc. of vigorous cultures of Az. vinelandii and Az. chroococcum.
6. Incubate 6–7 days at 20° C.
7. Add 0.7 gm. Hg. and 20 cc. H_2SO_4 and determine the nitrogen present by the regular Kjeldahl method.
8. Determine thus the effect of varying reaction of the medium on the nitrogen fixed by the pure cultures.

EXERCISE XXXIX.

THE INFLUENCE OF IRON; OF PHOSPHATE; AND OF AERATION ON THE FIXATION OF NITROGEN BY AZOTOBACTER.

1. Prepare 800 cc. of "Mannite solution."
2. Prepare 200 cc. of the same solution not adding the $\text{Fe}_2(\text{SO}_4)_3$.
3. Prepare similarly 200 cc. without the K_2HPO_4 .
4. Divide into 100 cc. portions in flasks and arrange as follows:

- 1 & 2—Regular "mannite" solution in 500 cc. Erlenmeyers.
- 3 & 4—Without $\text{Fe}_2(\text{SO}_4)_3$ solution in 500 cc. Erlenmeyers.
- 5 & 6—Without K_2HPO_4 solution in 500 cc. Erlenmeyers.
- 7 & 8—Regular "mannite" solution in 2,000 cc. Erlenmeyers.
- 9 & 10—Regular "mannite" solution in 1,000 cc. Erlenmeyers.
- 11 & 12—Regular "mannite" solution in 200 cc. Erlenmeyers.

5. Plug and sterilize in the autoclave as usual.
6. Inoculate each with 1 cc. of a vigorous culture of *Az. vinelandii*.

7. Incubate 6–7 days at 20°C .
8. Add 0.7 gm. Hg. and 20 cc. H_2SO_4 and determine the total nitrogen by the regular Kjeldahl method.
9. Determine the effect of the absence of $\text{Fe}_2(\text{SO}_4)_3$ and of K_2HPO_4 , and the effect of aeration on the fixation of nitrogen by *Azotobacter*.

EXERCISE XL.

THE INFLUENCE OF VARYING QUANTITIES OF SUGARS ON
NITROGEN-FIXATION BY AZOTOBACTER.

1. Make up 800 cc. of "mannite" solution as described, except that the mannite is not added.
2. Divide into eight 100 cc. portions in 500 cc. flasks.
3. Add sugar in the following amounts:

1—0.25% mannite	}	Az. vinelandii.
2—0.50% mannite		
3—1.00% mannite		
4—2.00% mannite		
5—0.25% mannite	}	Az. chroococcum.
6—0.50% mannite		
7—1.00% mannite		
8—2.00% mannite		

4. Plug and sterilize as usual in the autoclave.
5. Inoculate with 1 cc. of vigorous cultures of Az. vinelandii and Az. chroococcum.
6. Incubate 6–7 days at 20° C.
7. Add 0.7 gm. Hg. and 20 cc. H₂SO₄, and determine the nitrogen present by the regular Kjeldahl method.
8. Determine the effect of the varying amounts of sugar used on the nitrogen fixed by the pure cultures.

EXERCISE XLI.

THE INFLUENCE OF OTHER ORGANISMS ON NITROGEN-FIXATION BY AZOTOBACTER.

1. Measure off twelve 100 cc. portions of "mannite" solution in 500 cc. flasks.

2. Plug and sterilize as usual.

3. Inoculate with *Azotobacter* and pure cultures of other soil organisms, using 1 cc. of vigorous cultures in every case.

4.

1 & 2—*Az. vinelandii*.

3 & 4—*Az. vinelandii*—*B. mycoides*.

5 & 6—*Az. vinelandii*—*B. aerogenes*.

7 & 8—*Az. chroococcum*.

9 & 10—*Az. chroococcum*—*B. mycoides*.

11 & 12—*Az. chroococcum*—*B. aerogenes*.

5. Incubate 6–7 days at 20° C.

6. Add Hg. and H₂SO₄ and digest for total nitrogen by the regular Kjeldahl method.

7. The effect of the non-fixers on the fixation by *Azotobacter* is thus determined.

EXERCISE XLII.

ANAEROBIC NITROGEN FIXATION.

1. Measure off eight 100 cc. portions of Winogradski's solution in 500 cc. flasks.
2. To four of these add 10 mgs. $(\text{NH}_4)_2\text{SO}_4$.
3. Plug and sterilize as usual.
4. Inoculate with 10 cc. (= 5 gms. soil) of 5-minute soil infusions.
5. Arrange thus:
 - 1 & 2—Infusion Soil 1.
 - 3 & 4—Infusion Soil 1 + 10 mgs. $(\text{NH}_4)_2\text{SO}_4$.
 - 5 & 6—Infusion Soil 2.
 - 7 & 8—Infusion Soil 2 + 10 mgs. $(\text{NH}_4)_2\text{SO}_4$.
6. Incubate 10–14 days in a Novy jar or some other apparatus suitable for maintaining anaerobic conditions.
7. Remove and digest by the regular Kjeldahl method for total nitrogen.

EXERCISE XLIII.

THE ISOLATION AND DESCRIPTION OF CLOSTRIDIUM
PASTEURIANUM.

1. By means of a platinum loop make transfers from the cultures obtained in the previous exercise (XLII.) into tubes of sterile Winogradski's solution.

2. Incubate under anaerobic conditions for 10–14 days at 20° C.

3. Inoculate from the cultures obtained thus into tubes of sterile water and from these dilutions into two tubes (in series) of Winogradski's agar, liquefied and cooled (40° C.) and plate in the usual way.

4. Incubate under anaerobic conditions for 7–10 days at 20° C.

5. Inoculate from several well-isolated colonies into tubes of Winogradski's solution and incubate as before.

6. If the cultures are not pure they should be plated out as before until pure cultures are obtained.

7. Describe the character of the growth on the various media employed in the ordinary cultural examination of organisms.

8. Examine in stained and hanging-drop preparations for morphological descriptions.

GROUP VI. SYMBIOTIC NITROGEN FIXATION.**Exercises XLIV. to XLVI.****EXERCISE XLIV.****THE ISOLATION AND EXAMINATION OF *B. RADICICOLA* FROM
DIFFERENT LEGUMES.**

1. Thoroughly wash the roots of several legumes (*e. g.*, red clover, alfalfa, sweet clover, cowpeas, etc.), under the tap.

2. Compare the size, numbers and location of the nodules on the roots of these different legumes.

3. Remove the nodules from each, wash under the tap and immerse for 3 minutes in mercuric chloride solution (1:500).

4. With flamed forceps remove the nodules and take up the excess of the solution between the folds of filter paper wrung out of the same solution.

5. Then each nodule is held in flamed and cooled forceps and an incision made in it by means of a sterile knife or a chisel-edged platinum needle.

6. A sterile platinum needle is thrust into the opened nodule and rotated gently to cause some of the crushed tissue to adhere to it.

7. Then tubes of sterile "*Radicicola*" solution are inoculated.

8. After incubation agar plates are made in the usual way and the growth described.

9. Hanging-drop and stained preparations are made and the organisms carefully described.

10. Note any differences among the organisms from the different legumes.

EXERCISE XLV.

THE EFFECT OF INOCULATION AS SHOWN BY THE GROWTH OF
LEGUMES IN TUBES.

1. Thoroughly wash the seeds of the legumes to be used (alfalfa, red clover, sweet clover, etc.) and immerse in a mercuric chloride solution (1:500) for 5 minutes.

2. Place between the folds of filter paper wrung out of the same solution, and put in a warm place until dry.

3. Then drop one or two seeds of each legume into a large tube containing a loose mass of filter paper covered with 10–20 cc. of “Radicicola” solution.

4. Arrange thus:

1 & 2—Alfalfa.

3 & 4—Alfalfa, inoculated with alfalfa organisms.

5 & 6—Alfalfa, inoculated with sweet clover organisms.

7 & 8—Sweet Clover, inoculated with alfalfa organisms.

9 & 10—Sweet Clover, inoculated with sweet clover organisms.

11 & 12—Red Clover, inoculated with alfalfa organisms.

5. The seeds used are inoculated according to the plan given in (4) from pure cultures of the organisms obtained in the previous exercise.

6. After growth has taken place examine carefully for nodule formation and note the location, numbers and size of the nodules formed.

7. Describe the general effects of cross-inoculation, comparing the cross inoculated with the straight inoculated plants.

EXERCISE XLVI.

THE GROWTH OF B. RADICICOLA ON VARIOUS CULTURE MEDIA.

1. Inoculate from the pure cultures of B. Radicicola obtained from the various legumes in Exercise XLIV into tubes of the following solutions:

- (1) "Radicicola" solution.
- (2) "Sugar-ash" solution.
- (3) "Modified Radicicola" solution.
- (4) "Radicicola" solution + .1 gm. $(\text{NH}_4)_2\text{SO}_4$.

2. Incubate all the cultures 10-14 days at 20° C.

3. Describe the growth of the organisms in the different media.

4. Examine in hanging-drop and stained preparations and compare the size, motility, etc., of the organisms grown under these varying conditions.

EXERCISE XLVII.

THE CULTIVATION OF *AMYLOBACTER* SPECIES.

1. Boil 100 cc. of water in a tall, narrow beaker, until the air is expelled.

2. Add in small quantities at a time, coarsely powdered rice, or coarsely powdered barley.

3. When the mass is thick enough to hold a spoon in an upright position, stir in a small quantity of flour and incorporate it with the lower portion of the mass.

4. Cover with a glass plate and keep at 35°–37° C.

5. Fermentation will set in at 12–36 hours.

6. By staining with a dilute potassium iodide solution, granulose may be detected in many spindle-shaped sporangia.

EXERCISE XLVIII.

BUTYRIC FERMENTATION.

1. Six 250 cc. Erlenmeyer flasks are filled almost full of "synthetic" solution.
2. Plug and sterilize as usual.
3. Inoculate thus:

1 & 2—0.5 cc. old milk.
3 & 4—0.5 gm. old cheese.
5 & 6—0.5 gm. old compost.

4. Incubate 10–14 days at 20° C.
- 5 Test qualitatively for butyric acid.
6. Examine the cultures obtained, in hanging-drop and stained preparations and describe the organisms present.

EXERCISE XLIX.

AEROBIC CELLULOSE FERMENTATION.

1. Make up a solution containing:

1,000 cc. tap water.

1 gm. NH_4Cl .

0.5 gm. K_2HPO_4 .

2. Weigh off three portions of 1–2 gms. filter paper.

3. Place a piece of filter paper and 2.0 gms. CaCO_3 in each of three large Petri dishes. Pour enough of the above solution into each dish to cover the bottom completely.

4. Add 2 cc. of an infusion of well-rotted manure to one portion, and 2 gms. of garden soil to another portion.

5. Open every second day and add water to maintain the supply of moisture.

6. Incubate 3–4 weeks at 28° – 35° C.

7. Remove the filter paper very carefully with forceps and wash thoroughly.

8. Dry carefully and weigh.

9. The differences between the initial and final weights will show the amount of filter paper decomposed by the aerobic cellulose ferments.

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3. 421

EXERCISE L.

CELLULOSE FERMENTATION BY CULTURES OF DENITRIFIERS.

1. Make up the following solution:

1,000 cc. tap water.

2.5 gms. KNO_3 .

0.5 gm. K_2HPO_4 .

2. Place in Erlenmeyer flask of 200 cc. capacity 2 gms. of pure absorbent cotton; fill to the neck with the above solution; add a few cc. of ditch water.

3. Incubate at 35° for 15–20 days.

4. Test for the disappearance of nitrate reaction.

5. Pour off liquid; wash, dry and weigh the cotton.

6. Record changed appearance and loss in the weight of the cotton.

EXERCISE LI.

ANAEROBIC CELLULOSE FERMENTATION.

1. Make up a solution of:

1,000 cc. distilled water.

1 gm. K_2HPO_4 .

1.0 gm. $(NH_4)_3(PO_4)_2$.

0.5 gm. $MgSO_4$.

Traces of NaCl and Fe_2Cl_6 .

2. Place in 200 cc. Erlenmeyer flask 2 gms. of filter paper, or of pure absorbent cotton, and 2 gms. of $CaCO_3$.

3. Fill to the neck with the above solution.

4. Inoculate with several cc. of infusion of well-rotted horse manure.

5. Incubate at 30° – 35° for 4–6 weeks.

6. Pour off liquid; dry and weigh the cellulose.

7. Record changed appearance and loss in weight.

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EXERCISE LII.

THE ISOLATION AND DESCRIPTION OF CELLULOSE FERMENTS.

1. From the cultures obtained in Exercises XLIX. and L. inoculate by means of a platinum loop into tubes of sterile water.

2. Then inoculate into tubes of sterile "cellulose" agar in series and plate in the usual way.

3. Incubate 10-14 days at 20° C.; the cultures obtained from Exercise L. being kept under anaerobic conditions in a Novy jar.

4. From well isolated colonies inoculate into tubes of sterile "cellulose" solution containing strips of filter paper, tubes almost full of solution being used for the anaerobic ferments.

5. Microscopic hanging, drop and stained preparations are made and careful comparative descriptions of the organisms recorded.

EXERCISE LIII.

THE FORMATION OF ORGANIC ACIDS.

1. Measure off four 100 cc. portions of "synthetic" solution in 150 cc. Erlenmeyer flasks. Add to each portion 1 drop of a 1% solution of Fe_2Cl_6 and 2 or 3 drops of a 1% solution of CaCl_2 .

2. Plug and sterilize in the autoclave.

3. Prepare 400 cc. of the similarly modified "synthetic" solution using KNO_3 instead of peptone.

4. Measure off four 100 cc. portions of this solution in 150 cc. Erlenmeyers; plug and sterilize as usual.

5. Arrange thus:

1 & 2—"Synthetic" solution—*Az. vinelandii*.

3 & 4—"Synthetic" solution—*Az. chroococcum*.

5 & 6—"Synthetic" solution using KNO_3 —*B. mycoides*.

7 & 8—"Synthetic" solution using KNO_3 —*B. subtilis*.

6. Inoculate with 1 cc. of vigorous cultures of the above named organisms.

7. Incubate 10–14 days at 20°C .

8. Test qualitatively for acetic, butyric, and lactic acids.

EXERCISE LIV.

HUMUS FORMATION.

1. Weigh off ten 100 gm. portions of sieved soil, in tumblers.

2. Arrange thus:

1 & 2—nothing.

3 & 4—5 grams wheat straw (ground).

5 & 6—5 grams alfalfa hay (ground).

7 & 8—5 grams wheat straw (ground) + 1 gm. CaCO_3 .

9 & 10—5 grams alfalfa hay (ground) + 1 gm. CaCO_3 .

3. Stir in these materials thoroughly by means of a sterile spatula.

4. Add 12 cc. of sterile water to permit of the presence of organic matter containing 70% water.

5. Determine the moisture content of the soil employed and add sufficient sterile water to bring the water content up to the optimum.

6. Cover with Petri dish covers, weigh, and record the weight on the tumblers.

7. Incubate 3–4 months at 20°C .

8. Weigh every 10–14 days and add enough sterile water to keep the moisture conditions constant.

9. At the end of the incubation period determine the humus present in each tumbler by the usual method.

10. Determine the humus present in the soil used as the medium.

11. The difference between the amount of humus at the beginning and at the end of the experiment gives the quantity formed from the organic matter supplied.

EXERCISE LV.

SULFAT REDUCTION.

1. Fill six 150 cc. Erlenmeyer flasks almost full of "sulfat reduction" solution.

2. Plug and sterilize as usual.

3. Inoculate thus:

1 & 2—nothing.

3 & 4—1 cc. of ditch water.

5 & 6—2 cc. (= 1 gm. soil) of garden soil.

4. Incubate 7-14 days at 20° C.

5. At the end of that time test for sulfats by adding a little BaCl_2 solution to a portion of the culture liquid in a test tube. A white precipitate indicates the presence of sulfats—the absence of a precipitate shows complete sulfat reduction. Partial reduction is indicated by the darkening of the solution due to the formation of sulfid of iron.

No reduction seen from

APPENDIX A.

PREPARATION OF MEDIA.

I. *Bouillon*.

1. To 1 liter (1,000 cc.) of water add the following substances:

- 10 gms. peptone.
- 5 gms. NaCl.
- 5 gms. Liebig's beef extract.

2. Boil until dissolved and restore to the original volume with water.

3. Titrate thus: By means of a pipette, transfer 5 cc. of the solution to a porcelain dish. Add 45 cc. of distilled water and heat to boiling. Titrate while hot with $\frac{N}{20}$ NaOH using phenolphthalein as an indicator. A faint pink color indicates the end point. The reaction of the medium is expressed in degrees of Fuller's scale, which represent the number of cc. of acid or alkali present in 1,000 cc. of the medium. A minus (—) sign indicates alkalinity, and a plus (+) sign acidity; *e. g.*, -20° would indicate that the medium was twenty degrees alkaline or that 20 cc. of normal acid must be added per liter to neutralize it.

The reaction of the bouillon is to be $+ 10^{\circ}$ Fuller's scale or 1% acid and it must be adjusted to that point by adding normal NaOH or HCl as required; *e. g.*, suppose 30 cc. of $\frac{N}{20}$ NaOH were required to neutralize 5 cc. of the medium, then 100 cc. would require 3.0 cc. of normal NaOH, or a liter would require 30 cc. of normal NaOH. The bouillon in this case would be 30° acid. The optimum reaction, however, being 10° acid, 20 cc. of normal NaOH must be added per liter of bouillon.

4. Boil vigorously for five minutes or until the sediment becomes flaky.
5. Make up with water any loss by evaporation.
6. Filter hot through folded filter paper.
7. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

II. *Bouillon Agar.*

1. Make a paste of 20 gms. of powdered agar in cold water and add to 1,000 cc. of bouillon.
2. Boil until dissolved.
3. Filter through absorbent cotton.
4. Fill into test tubes taking care not to allow the medium to touch the necks of the tubes as this will cause the cotton to stick to the glass when the plugs are removed.
5. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

III. *Gelatin.*

1. Dissolve 10 gms. of gelatin in 100 cc. of bouillon (boiling hot). Do not boil after the gelatin has been added or the solidifying power may be destroyed.
2. Filter through filter paper.
3. Sterilize in the autoclave at 10 lbs. pressure for ten minutes.

IV. *Potatoes.*

1. Wash the potatoes thoroughly with water.
2. By means of a borer cut out cylinders 3-4 inches in length.
3. Remove the skin and cut each cylinder into two wedge-shaped pieces.
4. Place a small piece of cotton in the bottom of each test tube and moisten it. This is to prevent the potato from drying out.
5. Rinse the wedges thoroughly in water and place one in each test tube.
6. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

V. "*Mannite*" *Solution for Nitrogen-fixers.*

1. To one liter (1,000 cc.) of distilled water add the following substances:

0.2 gm. K_2HPO_4 .
0.2 gm. $MgSO_4$.
0.02 gm. $CaCl_2$.
1 drop 10% sol. Fe_2Cl_6 .
15.0 gms. mannite.

2. Stir until all the substances are dissolved.
3. Add a few drops of phenolphthalein and then by means of a pipette add 10% NaOH, until the solution assumes a very faint pink color.
4. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

VI. "*Mannite*" Agar.

1. Make a paste of 20 gms. of powdered agar in water and add to 1,000 cc. of "mannite" solution.
2. Boil until dissolved.
3. Filter through absorbent cotton.
4. Sterilize in the autoclave as usual.

VII. "*Giltay*" Solution for Denitrification.

1. Dissolve the following substances in 800 cc. of distilled water: (Solution I.)

2 gms. KH_2PO_4 .
2 gms. MgSO_4 .
1 gm. KNO_3 .
0.2 gm. CaCl_2 .
2 drops sol. Fe_2Cl_6 .
5 gms. citric acid.

2. Add a few drops of phenolphthalein and then by means of a pipette drop in just enough 10% NaOH to turn the solution a faint pink color.

3. Dissolve 10 gms. dextrose in 200 cc. of distilled water (Solution II).

4. Mix solutions I. and II. very thoroughly.

5. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

VIII. "*Giltay*" Agar.

1. Make a paste of 20 gms. of powdered agar in water and add to 1,000 cc. of "*Giltay*" solution.
2. Boil until dissolved.
3. Filter through absorbent cotton.
4. Sterilize in the autoclave as usual.

IX. "*Solution for Nitrite Formation.*"

1. Dissolve the following materials in 1,000 cc. of distilled water.

1 gm. $(\text{NH}_4)_2 \text{SO}_4$.
1 gm. KH_2PO_4 .
0.5 gm. MgSO_4 .
0.4 gm. $\text{Fe}_2(\text{SO}_4)_3$.
2 gms. NaCl.

2. Stir until the solution is complete.
3. Measure off 100 cc. portions in flasks.
4. Add 1 gm. CaCO_3 to each portion.
5. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

X. "*Nitrite Solution for Nitrification.*"

1. Dissolve in 1 liter (1,000 cc.) of distilled water:

1 gm. NaNO_2 .
1 gm. Na_2CO_3 .
0.5 gm. KH_2PO_4 .
0.5 gm. NaCl.
0.4 gm. $\text{Fe}_2(\text{SO}_4)_3$.
0.3 gm. MgSO_4 .

2. Stir until all the materials are in solution.
3. Sterilize in the autoclave as usual.

XI. "*Nitrate-Nitrogen Transformation*" Solution.

1. Dissolve in 1 liter (1,000 cc.) of distilled water:

0.2 gm. K_2HPO_4 .
0.2 gm. $MgSO_4$.
0.5 gm. $NaNO_3$.
0.02 gm. $CaCl_2$.
1.0 gm. dextrose.
1 drop. sol. Fe_2Cl_6 .

2. Stir until dissolved.

3. Add a few drops of phenolphthalein and then 10% NaOH until the solution is just neutral.

4. Sterilize in the autoclave at 15 lbs. pressure for ten minutes.

XII. "*Ammonia-Nitrogen Transformation*" Solution.

1. Dissolve in 1 liter of distilled water:

0.2 gm. K_2HPO_4 .
0.2 gm. $MgSO_4$.
1.0 gm. $(NH_4)_2 SO_4$.
0.02 gm. $CaCl_2$.
1.0 gm. dextrose.
1 drop sol. Fe_2Cl_6 .

2. Stir until dissolved.

3. Add 10% NaOH until the solution is neutral to phenolphthalein.

4. Sterilize in the autoclave as usual.

XIII. "*Synthetic*" Solution.

1. To 1,000 cc. of tap water add the following:

10 gms. dextrose.
0.2 gm. MgSO_4 .
0.5 gm. K_2HPO_4 .
0.05 gm. peptone.

2. Stir until dissolved.
3. Sterilize in the autoclave as usual.

XIV. "*Synthetic*" Agar.

1. Make a paste of 20 gms. of powdered agar with cold water and add to 1,000 cc. of "Synthetic Solution."
2. Boil until dissolved.
3. Filter through absorbent cotton.
4. Sterilize as usual.

XV. "*Cellulose*" Solution.

1. Dissolve in 1,000 cc. of tap water:

0.5 $(\text{NH}_4)_3\text{PO}_4$.
0.5 gm. KH_2PO_4 .
0.1 gm. MgSO_4 .
0.1 gm. NaCl .

2. Sterilize in the autoclave as usual.

XVI. "*Radlicola*" Solution.

1. Dissolve in 1,000 cc. of tap water:

10 gms. saccharose.
1 gm. K_2HPO_4 .

2. Stir until dissolved.
3. Sterilize in the autoclave at 10 lbs. pressure for 20 minutes.

XVII. "*Radlicola*" Agar.

1. Make a paste of 20 gms. of powdered agar in cold water and add to 1,000 cc. of "*Radlicola*" solution.
2. Boil until dissolved.
3. Filter through absorbent cotton.
4. Sterilize in the autoclave for twenty minutes at 10 lbs. pressure.

XVIII. "*Urea Solution*."

1. To bouillon prepared as in I. add 1% urea and boil until dissolved.
2. Sterilize in the autoclave as usual.

XIX. "*Urea Agar*."

1. Make a paste of 20 gms. of powdered agar in cold water and add to 1,000 cc. of "*Urea Solution*."
2. Boil until dissolved.
3. Filter through absorbent cotton and sterilize as usual.

XX. "*Sugar-ash*" Solution For *Radicalicola*.

1. To 1 liter (1,000 cc.) of distilled water, add 15 gms. of wood-ashes (beech, elm, maple).
2. Heat in steam, boil one minute and filter through moistened filter paper.
3. To the filtrate add 10 gms. Maltose and stir until dissolved.
4. Sterilize in the autoclave at 10 lbs. pressure for 20 minutes.

XXI. "*Sugar-ash*" Agar.

1. Make a paste of 20 gms. of powdered agar in cold water and add to 1,000 cc. of "sugar-ash" solution.
2. Boil until dissolved.
3. Filter through absorbent cotton.
4. Sterilize in the autoclave at 10 lbs. pressure for 20 minutes.

XXII. *Omelianski's Magnesia Gypsum Blocks For Cultivating the Nitrifying Organisms.*

1. One per cent. carbonate of magnesia is uniformly mixed with gypsum and water is added with constant stirring until the mass assumes the consistency of sour cream.
2. Pour out on plate glass and spread out.
3. As soon as the mass becomes of a doughy consistency, and is ready to harden, circular pieces for Petri dishes, or strips for test tubes, are cut out.
4. When the gypsum plate has become hard, it is pried loose from the glass and placed bottom up in the Petri dishes.

5. Enough of the solution for "nitrite formation" is added to half cover the block.

6. Sterilize in the autoclave at 15 lbs. pressure for 10 minutes.

XXIII. *Winogradski's Medium for Nitrogen-Fixation.*

1. To 1,000 cc. distilled water add the following substances:

- 1.0 gm. K_2HPO_4 .
- 0.5 gm. $MgSO_4$.
- 0.01 gm. NaCl.
- 0.01 gm. $Fe_2(SO_4)_3$.
- 0.01 gm. $MnSO_4$.
- 20 gms. dextrose.
- 30 gms. $CaCO_3$.

2. Stir until all the substances except the $CaCO_3$ are in solution.

3. Sterilize in the autoclave as usual.

XXIV. *Winogradski's Agar.*

1. Make a paste of 20 gms. of powdered agar in cold water and add to 1,000 cc. of the solution XXIII.

2. Boil until dissolved.

3. Filter through absorbent cotton.

4. Sterilize in the autoclave as usual.

XXV. *Winogradski's Silicate Jelly.*

1. To 100 cc. HCl (sp. gr. 1.10° Beame's) is added drop by drop 100 cc. sodium silicate (sp. gr. 1.09) stirring constantly with a glass rod.

2. Place in collodion sacks and dialyze for 12 hours in running water.

3. The dialyzed silicate jelly is then poured out of the collodion sacks into a beaker and boiled for a few minutes to drive off the absorbed air.

4. Cool down to 50° C. and the following boiled and cooled solution is added:

0.4 gm. $(\text{NH}_4)_2\text{SO}_4$.

0.05 gm. MgSO_4 .

0.1 gm. K_2HPO_4 .

0.6 gm. Na_2CO_3 .

Trace CaCl_2 .

These substances are dissolved in as little water as possible and added to:

100 gm. silicate jelly.

5. Sterilize in the autoclave at 110° C. for 15 minutes.

XXVI. "*Modified Radicicola Solution.*"

1. To 1 liter (1,000 cc.) distilled water add the following:

5 gms. saccharose.

2 gms. K_2HPO_4 .

0.1 gm. MgSO_4 .

0.5 gm. NaCl.

2. Boil until dissolved.

3. Sterilize in the autoclave as usual.

XXVII. "*Solution for Sulfat Reduction.*"

1. Dissolve in 1,000 cc. of tap water :

0.5 gm. K_2HPO_4 .
5.0 gms. sodium lactate.
1.0 gm. asparagin.
1.0 gm. $MgSO_4$.
Trace ferrous sulfat.

2. Sterilize in the autoclave as usual.

XXVIII. *Sugar Broths.*

1. 1 gm. of any of the sugars (dextrose, maltose, saccharose, mannite, or lactose) is added to 100 cc. of bouillon.

2. Fermentation tubes are filled and sterilized in the steam sterilizer for 30 minutes on three consecutive days.

XXIX. *Dunham's Solution.*

1.

1,000 cc. distilled water.
10 gms. Witte's peptone.
5 gms. NaCl.

2. Fill into test tubes and sterilize in the autoclave as usual.

XXX. *Nitrate Solution.*

1.

0.5 gm. NaCl.
1.0 gm. peptone.
0.2 gm. KNO_3 .
1,000 cc. water.

2. Fill into test tubes and sterilize as usual.

XXXI. *Milk.*

1. The milk should be obtained from a clean dairy and if not free from cream should be filtered or separated.

2. 10 cc. portions in test tubes are sterilized in the steam sterilizer for 15 minutes on each of four consecutive days.

3. After standing a week the milk is ready for use.

XXXII. *Litmus Milk.*

1. Fresh separated milk is employed.

2. To each 100 cc. add 2 cc. of a saturated solution (1 gm.—15 cc. water) of blue litmus.

3. 10 cc. portions in test tubes are sterilized in the steam sterilizer for 15 minutes on four consecutive days.

APPENDIX B.

METHODS.

1. *Kjeldahl Method for Total Nitrogen.*

1. Reagents.

(a) Standard HCl.

Approximately one-half normal. The absolute strength is determined by precipitating with silver nitrate and weighing the silver chlorid formed.

(b) Standard Alkali (NH_4OH).

Approximately one-tenth normal. Standardize against the HCl.

(c) Potassium Sulfid solution.

40 gms. K_2S per liter of water.

(d) Sodium Hydroxid.

A saturated solution.

(e) Indicator.

3 gms. cochineal digested in a mixture of 50 cc. alcohol and 200 cc. distilled water for 2 days and filtered.

2. Determination.

(a) Digestion.

The substance to be analyzed is placed in a Kjeldahl flask with 0.7 gms. Hg. and 20 cc. H_2SO_4 and heated in an inclined position until all the organic matter is oxidized and the nitrogen changed to $(\text{NH}_4)_2\text{SO}_4$.

(b) Distillation.

After cooling transfer to distilling flasks with 200 cc. water and add 0.5 gm. zinc dust, 25 cc. K_2S solution and 50 cc. of $NaOH$. Connect the flask with the condenser and distil until all the ammonia has passed over, collecting it in the standard HCl and titrating against the standard alkali, using cochineal as an indicator.

II. *Phenolsulfonic Acid Method for Nitrates.*

1. Reagents.

(a) Phenolsulfonic acid.

15 gms. phenol dissolved in a mixture of 92.5 cc. H_2SO_4 and 7.5 cc. water.

(b) Standard Nitrate solution.

0.7221 gms. KNO_3 per liter of water. 25 cc. are evaporated to dryness and treated as described below; the solution made up to 100 cc. with water, then each cc. = .025 mg. nitrogen.

2. Determination.

The solution in which the nitrate is to be determined is evaporated to dryness on a water bath. 2 cc. of the phenolsulfonic acid is added and stirred thoroughly.

Stand 10–15 minutes.

Add 15 cc. of water and 10–15 cc. of ammonium hydroxid. Dilute to 100 cc. in a colorimeter tube and compare with the standard solution in another tube, diluting the unknown or the standard as necessary.

III. *Griess Colorimetric Method for Nitrites.*

1. Reagents.

(a) Sulfanilic acid.

0.5 gm. sulfanilic acid in 150 cc. of dilute acetic acid (sp. gr. 1.04).

(b) Naphthylamine acetate.

Boil 0.1 gm. of a naphthylamine in 20 cc. of water and filter through a plug of absorbent cotton into 180 cc. of dilute acetic acid (sp. gr. 1.04).

(c) Nitrite reagent.

Mix equal parts of the sulfanilic acid and naphthylamine acetate.

(d) Standard Sodium nitrite.

0.0836 gm. pure silver nitrite dissolved in water. Add a solution of NaCl until all the silver is precipitated. Make up to 250 cc., shake and stand until the precipitate has settled. Dilute 10 cc. of the supernatant liquid to 100 cc. with water. Each cc. of this = 0.01 mg. NO_2 .

(e) Standard colorimetric solution.

10 cc. of the above standard nitrite solution treated as described below and made up to 100 cc. with water.

2. Determination.

To the solution to be tested add 16 cc. of the nitrite reagent (c) and dilute to 100 cc. in a colorimeter tube, and compare with the standard colorimetric solution (e) diluting the one or the other as necessary.

IV. *Humus Determination.*

1. 10 gms. of the sample are placed in a Gooch crucible and extracted with 1% HCl until the filtrate gives no reaction with ammonia and ammonium oxalate.

2. The acid is removed by washing with water.

3. The contents of the crucible are then washed into a glass stoppered cylinder with 500 cc. of 4% NH_4OH . Stand 24 hours with occasional shaking. Stand 12 hours to permit settling.

4. The supernatant liquid is filtered and an aliquot evaporated, dried at 100°C . and weighed.

5. The residue is then ignited and weighed.

6. The humus is calculated from the difference in weight between the dried and the ignited residues.

V. *Ammonia Determination.*

10–15 gms. of heavy MgO and a piece of paraffin are added to the solution to be tested, the NH_3 distilled off, collected in standard acid, and titrated against standard alkali.

VI. *Some Qualitative Tests.*

1. Nitrates.

A small portion of the solution to be tested is treated with a solution of diphenylamine in H_2SO_4 . The presence of nitrates is indicated by the appearance of a blue color.

2. Acetic acid.

Treat a little of the solution to be tested in a test tube with a mixture of H_2SO_4 and ethyl alcohol. The presence of acetic acid may be detected by the ethyl acetate odor which is quite distinctive.

3. Butyric acid.

A small portion of the solution to be tested is treated as in the test for acetic acid and the presence of butyric acid is shown by the peculiar odor of the ethyl butyrate produced.

4. Lactic acid.

Add 10 cc. of Uffelmann's solution to a little of the culture in a test-tube. The formation of a canary yellow color indicates the presence of lactic acid.

(Uffelmann's solution: 100 cc. of a 1% solution of carbolic acid to which 10-15 drops of a solution of Fe_2Cl_6 have been added.)

APPENDIX C.

DESCRIPTIVE CHART—SOCIETY OF AMERICAN BACTERIOLOGISTS.

Cultural and morphological descriptions of organisms are to be made according to this chart and records kept according to the numerical system.

DESCRIPTIVE CHART—SOCIETY OF AMERICAN BACTERIOLOGISTS.

Prepared by F. D. Chester, F. P. Gorham, Erwin F. Smith, Committee on Methods of Identification of Bacterial Species.
Endorsed by the Society for general use at the Annual Meeting, Dec. 31, 1907.

GLOSSARY OF TERMS.

AGAR HANGING BLOCK, a small block of nutrient agar cut from a poured plate, and placed on a cover-glass, the surface next the glass having been first 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.

AMORPHIOUS, without visible differentiation in structure.

ARBORESCENT, 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 pseudo-zoogloae.

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

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

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

COPROPHYL, 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, same as **DIASTATIC**, 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 pseudozoogloae, *i. e.*, 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 gentian violet, methyl violet, &c. The + mark is to be given only when the bacteria are deep blue or remain blue after counterstaining with Bismark brown.

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 a membrane.

MINIMUM TEMPERATURE, temperature below which growth does not take place.

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

NAPIFORM, liquefaction with the form of a turnip.

NITROGEN REQUIREMENTS, 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

PSEUDOZOOGLOEAE, clumps of bacteria, not dissolving 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.

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.

REPAND, wrinkled.

RAPID, developing in 24 to 48 hours.

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

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

SLOW, requiring 5 or 6 days or more for development.

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

SPORANGIA, cells containing endospores.

SPREADING, growth extending much beyond the line of inoculation, *i. e.*, several millimeters or more.

STRATIFORM, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.

THERMAL DEATH-POINT, the degree of heat required to kill the young fluid cultures of an organism exposed for 10 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.

VERRUCOSE, growth wart-like, with wart-like prominences.

VERMIFORM-CONTOURED, growth like a mass of worms, or intestinal coils.

VILLOUS, growth beset with hair-like extensions.

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

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

NOTES.

- (1) For decimal system of group numbers see Table 1. This will be found useful as a quick method of showing close relationships inside the genus, but is not a sufficient characterization of any organism.
- (2) The morphological characters shall be determined and described from growths obtained upon at least one solid medium (nutrient agar) and in at least one liquid medium (nutrient broth). Growths at 37° C. shall be in general not older than 24 to 48 hours, and growths at 20° C. not

older than 48 to 72 hours. To secure uniformity in cultures, in all cases preliminary cultivation shall be practiced as described in the revised Report of the Committee on Standard Methods of the Laboratory Section of the American Public Health Association, 1905.

(3) The observation of cultural and bio-chemical features shall cover a period of at least 15 days and frequently longer, and shall be made according to the revised Standard Methods above referred to. All media shall be made according to the same Standard Methods.

(4) Gelatin stab cultures shall be held for 6 weeks to determine liquefaction.

(5) Ammonia and indol tests shall be made at end of tenth day, nitrate tests at end of fifth day.

(6) Titrate with $\frac{N}{20}$ NaOH, using phenolphthalein as an indicator: make titrations at same times from blank. The difference gives the amount of acid produced.

The titration should be done after boiling to drive off any CO₂ present in the culture.

(7) Generic nomenclature shall begin with the year 1872 (Cohn's first important paper).

Species nomenclature shall begin with the year 1880 (Koch's discovery of the poured plate method for the separation of organisms).

(8) Chromogenesis shall be recorded in standard color terms.

TABLE I.

A NUMERICAL SYSTEM OF RECORDING THE SALIENT CHARACTERS OF AN ORGANISM. (GROUP NUMBER.)

100.	Endospores produced
200.	Endospores not produced
10.	Aerobic (Strict)
20.	Facultative anaerobic
30.	Anaerobic (Strict)
1.	Gelatin liquefied
2.	Gelatin not liquefied
0.1	Acid and gas from dextrose
0.2	Acid without gas from dextrose
0.3	No acid from dextrose
0.4	No growth with dextrose
.01	Acid and gas from lactose
.02	Acid without gas from lactose
.03	No acid from lactose
.04	No growth with lactose
.001	Acid and gas from saccharose
.002	Acid without gas from saccharose
.003	No acid from saccharose
.004	No growth with saccharose
.0001	Nitrates reduced with evolution of gas
.0002	Nitrates not reduced
.0003	Nitrates reduced without gas formation
.00001	Fluorescent
.00002	Violet chromogens
.00003	Blue “
.00004	Green “
.00005	Yellow “
.00006	Orange “
.00007	Red “
.00008	Brown “
.00009	Pink “
.00000	Non-chromogenic
.000001	Diastasic action on potato starch, strong
.000002	Diastasic action on potato starch, feeble
.000003	Diastasic action on potato starch, absent
.0000001	Acid and gas from glycerine
.0000002	Acid without gas from glycerine
.0000003	No acid from glycerine
.0000004	No growth with glycerine

The genus according to the system of Migula is given its proper symbol, which precedes the number thus: (7)

BACILLUS COLI (Esch.) Mig.	becomes B.	222.111102
BACILLUS ALCALIGENES Petr.	“ B.	212.333102
PSEUDOMONAS CAMPESTRIS (Pam.) Sm.	“ Ps.	211.333151
BACTERIUM SUICIDA Mig.	“ Bact.	222.232203