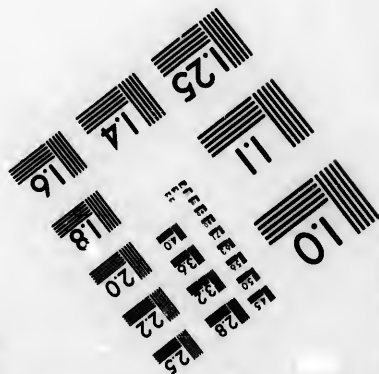
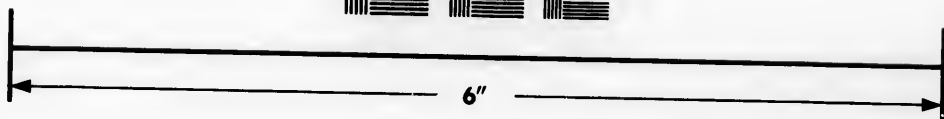
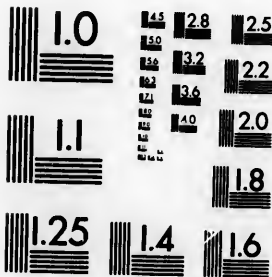


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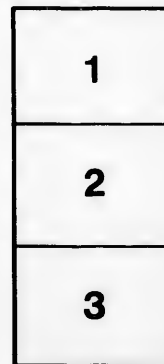
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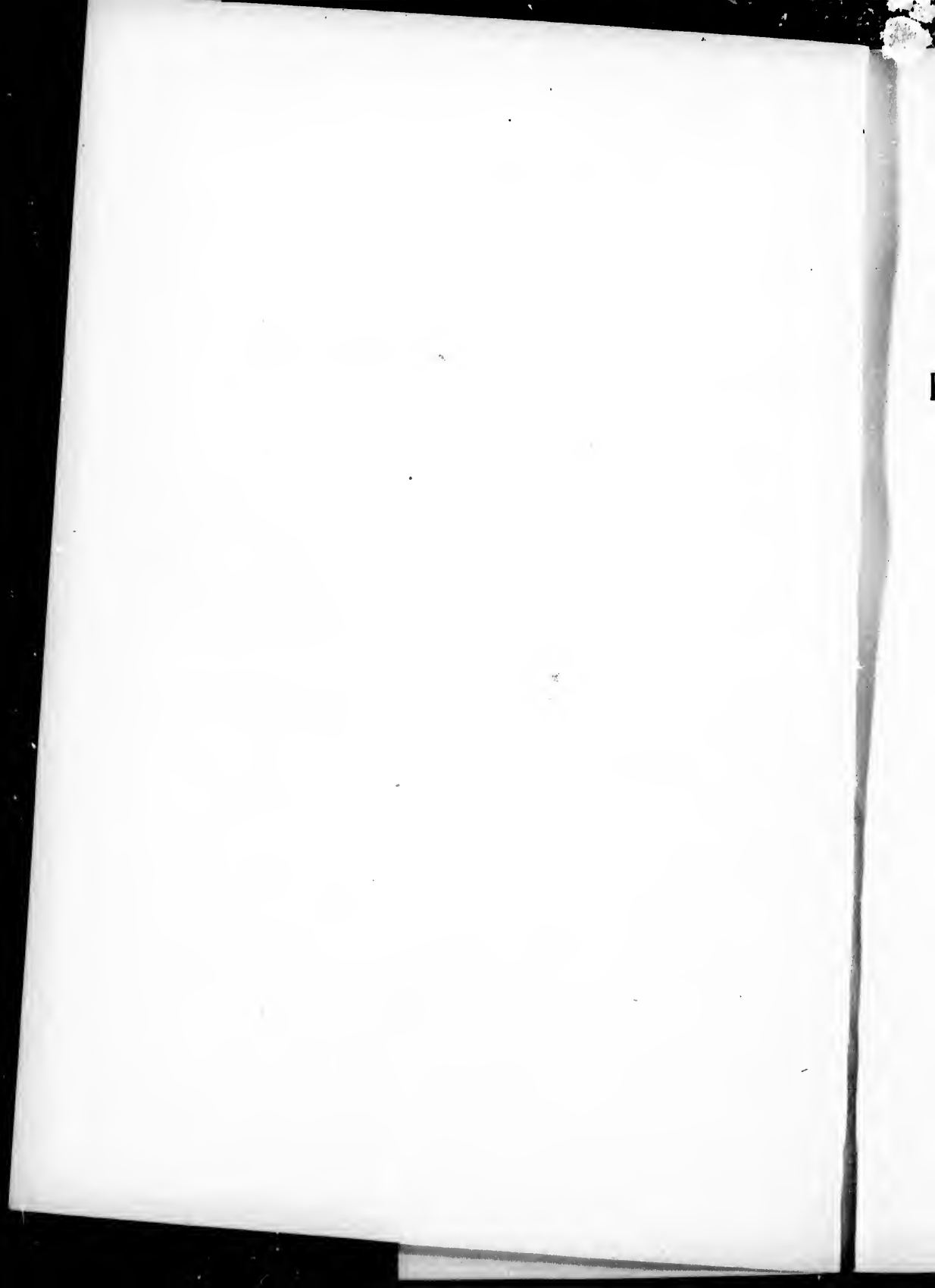
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A LABORATORY GUIDE
IN
PRACTICAL BACTERIOLOGY

WITH AN OUTLINE FOR THE CLINICAL EXAMINATION
OF THE URINE, BLOOD AND GASTRIC CONTENTS,

BY
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PATHOLOGIST TO THE GENERAL HOSPITAL, KINGSTON.

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PREFACE.

This book has been prepared for the guidance of students taking the practical course in Bacteriology. Much time and labor have hitherto been expended in the writing out, both by the lecturer and the student, of the daily work of the class, and to obviate this it was thought best to place these notes in an accessible and connected form. The usual course covers a period of from eight to ten weeks, and comprises a series of twenty demonstrations, of at least two hours each.

There are already several excellent handbooks for the guidance of students, notably that of Kanthack and Drysdale. As the author worked under the former's guidance, their methods have been used as a basis. Free use has also been made of methods advised by, or acquired from others, which have been found satisfactory.

In Part II the preparation of culture media, methods of sterilization of bacteriological utensils, and the methods followed in water and milk analysis are considered.

The author has appended in Part III the plan of work followed in the usual course in clinical microscopy and diagnosis, covering that elementary work, with which every physician should now not only be cognizant, but employ.

W. T. CONNELL.

KINGSTON, ONTARIO,
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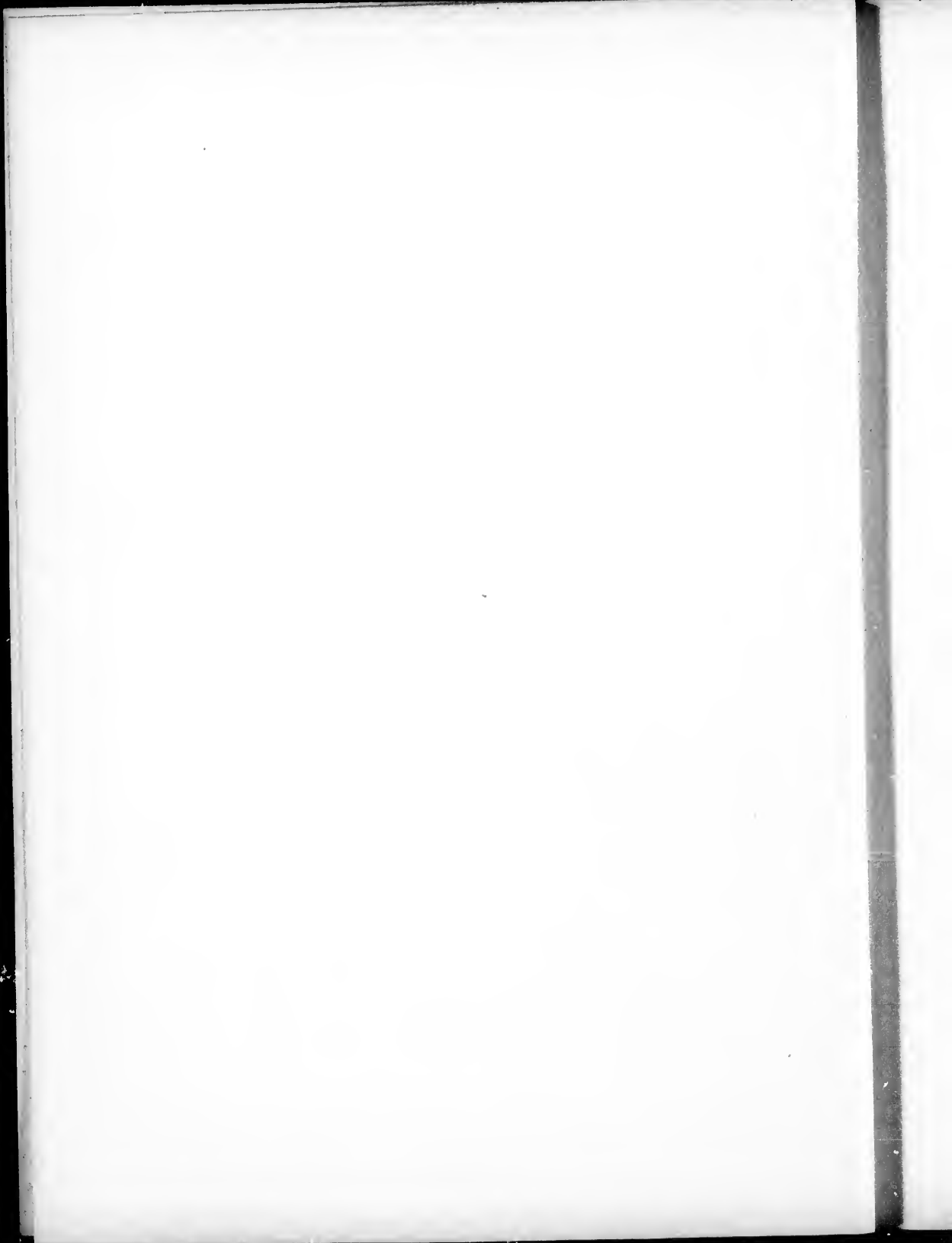


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PART I.

PRACTICAL BACTERIOLOGY.

DEMONSTRATION I.

Inoculation of Culture Media from Pure Cultures of Bacteria.

Students must remember that they are to work with many of the actual causative factors of disease—the pathogenic bacteria; hence there is a liability to self-infection or infection of the laboratory, unless care is taken to develop a proper technique. The practical work is started with non-pathogenic species, or with species of low pathogenic power, so that such a technique may be acquired without danger.

The points to be noted by the student are:

- (a) The character of the various culture media.
- (b) The method of holding culture tubes, and removing and replacing plugs during inoculation.
- (c) The care, and particularly the sterilization, of the platinum needle or loop, before and after use.
- (d) That minute amounts of the bacterial culture suffice for inoculation purposes.
- (e) The proper labelling and placing in incubators of the inoculated tubes.

1. Make two agar-agar slope cultures of *Bacillus prodigiosus*.

Place one tube in the incubator at 37°C (98.6°F), and the other at 20°C (69°F).

Examine these tubes at the end of 24 and 48 hours. Growth is more rapid at the higher temperature, but the pigmentation is slight, while a more slowly appearing growth, deeply pigmented, is noted in the other tube.

Pigmentation is in nearly all chromogenic species, more marked at the lower temperature than at the higher.

2. Make a gelatine slope culture of *Bacillus cyanogenus* and place in the incubator at 20°C.

Examine each day and note the gradual intensification of the pigmentation of the media, the growth itself being uncolored.

3. Make two potato tube cultures of *Bacillus ruber*.

Keep one exposed freely to light in the room, and place the second in the incubator (dark) at 20°C. Growth and pigmentation will be more marked in the latter tube.

4. Make gelatine stab cultures of (a) *Bacillus pyocyaneus*, (b) *Staphylococcus pyogenes aureus*, (c) *Sarcina lutea*.

Incubate at 20°C and examine daily. These bacteria liquefy gelatine more or less rapidly.

5. Make broth cultures of (a) *Torula rosea* and (b) *Bacillus proteus mirabilis*. Place in incubator at 20°C.

6. Make gelatine slope cultures of (a) *Staphylococcus cereus flavus*, (b) *Streptococcus pyogenes*. Keep in incubator at 20°C.

DEMONSTRATION II.

Staining and examination of pure cultures of Bacteria
from liquid media.

The stains which are ordinarily used are:

(a) Gentian violet. This stain is used in several forms. Of these anilin water, gentian violet is most used. It consists of,

Anilin oil	4 cc.
Distilled water	100 cc.
Shake thoroughly, filter and add saturated alcoholic solution of gentian violet	11 cc.

This stain ought to be freshly prepared every two or three weeks.

Gentian violet may also be used in a diluted alcoholic solution, and in this form is one of the most satisfactory stains for use in pure culture staining. It is made at time of using by adding two or three drops of filtered saturated alcoholic solution of gentian violet to a watch glass of distilled water.

(b) Methylene blue. This may be used in several forms. The stain most commonly employed is Loeffler's alkaline solution. Its composition is,

Saturated alcoholic solution of methylene blue	30 cc.
$\frac{1}{10000}$ caustic potash solution	100 cc.

(c) Fuchsin. Several staining solutions are used of this dye, of these Ziehl's carbol-fuchsin is most useful. This stain is made by taking,

Saturated alcoholic solution of fuchsin	
crystals	11 cc.
5% carbolic acid solution	89 cc.

Fuchsin can also be used as anilin water fuchsin, or as diluted fuchsin. These are made of the same strength as the like preparations of gentian violet.

All stains must be filtered before use.

Cover glasses should be kept in alcohol and handled with forceps. When they are required for use, rub dry with a clean cloth.

1. (a) Take a clean cover glass and sterilize it by passing it quickly through the gas (or spirit lamp) flame. Then with a platinum loop (using all the precautions of the first lesson), remove a drop of the liquefying gelatine culture of *Staphylococcus pyogenes aureus*, and smear this over the cover glass.

(b) Allow the film to dry in the air, or dry it by holding it high over the flame. Fix the film by passing it quickly three times through the flame.

(c) Clear away the gelatine by placing the cover glass in 10% acetic acid for five minutes.

(d) Remove from the acid, rinse through water and then dry.

(e) Float the cover glass, film surface down, in anilin water gentian violet, or in diluted gentian violet, for

one to two minutes. If desired, the stain can be dropped on the cover glass.

(f) Remove and wash in water; dry thoroughly and mount the cover glass in Canada balsam.

Examine the specimen with a $\frac{1}{2}$ oil-immersion lens, using the plane mirror and open diaphragm, with the Abbé condenser.

Use of Oil-Immersion Lens.

In using the oil-immersion lens, first place a drop of immersion oil on the centre of the film to be examined, and placing the preparation on the centre of the microscopic stage, fix one end of the slide with a clip. Lower the lens with the coarse adjustment, till it touches the drop. With the eye to the ocular, in stained preparations, lower very slowly till the field becomes colored. In unstained preparations always use the fine adjustment after the lens touches the oil. Now, with the fine adjustment carefully lower the lens till the field comes clearly into view. The slide can be readily moved about at the unclipped end. Before removing slide always raise the objective well out of the oil with the coarse adjustment.

2. Examine in like manner the liquefying gelatine cultures of *Bacillus pyocyaneus* and of *Sarcina lutea*, substituting Loeffler's methylene blue as staining agent, the films being left in the stain from two to three minutes.

3. Examine in like manner the broth culture of *Torula rosea*, using Loeffler's methylene blue as stain.

One-half to one minute will suffice for staining, as torulae stain rapidly as a rule.

4. Prepare in like manner a cover glass film of the broth culture of *Bacillus proteus mirabilis*. Stain with carbol-fuchsin, one-quarter to one-half minute, wash, dry and mount in Canada balsam.

DEMONSTRATION III.

Examination of pure cultures of Bacteria grown on solid media—
Gram's method of staining.

1. With a sterile platinum loop, place a drop of sterile distilled water on a cover glass. Remove then, with due precautions, a minute amount of *Bacillus prodigiosus*, and smear with the water, carefully over the cover glass surface. Dry and pass three times through the flame. As a rule no clearing in acetic acid will be needed in examining bacteria from solid media. Float the cover glass in anilin gentian violet for one to two minutes. Wash in water, dry and mount in Canada balsam.

2. Examine in like manner the potato culture of *Bacillus ruber* and the gelatine culture of *Bacillus cyanogenus*. Use either Loeffler's methylene blue or diluted gentian violet for staining.

Gram's Method.

Gram's method of staining is based on the fact that, when some bacteria are stained with certain dyes (of which anilin gentian violet is the principal), and then treated with a solution of iodine, the iodine fixes the stain so that it is not washed out by after treatment with alcohol. It affords a good differential stain, as some species stain, while others do not. Various modifications will be noted in this method, for the staining of pus and tissues.

3. Prepare cover glass film from the gelatine culture of *Streptococcus pyogenes*, and also from the liquefying gelatine culture of *Staphylococcus pyogenes aureus*. Stain them according to Gram's method as follows:

(a) Place the films in alcohol one to two minutes.

(b) Without drying, transfer to anilin gentian violet for two minutes.

(c) Rinse in water or .6% saline solution, and then place for one-half to one minute in Gram's iodine solution, (Iodine 1 part, Potassium iodide 2 parts, Water 300 parts).

(d) Rinse in water, and then in alcohol, till no more stain comes away.

(e) Wash in water, dry and mount in Canada balsam.

DEMONSTRATION IV.

Examination of Bacteria unstained—Hanging Drops—
Hanging Drop Cultures.

1. With a sterile loop remove a drop of the liquefied gelatine culture of *Staphylococcus pyogenes aureus*, and place on a cover glass. Invert this cover glass on a slide.

Examine with the $\frac{1}{12}$ oil-immersion lens, shutting off nearly all the light; or examine, after removing the Abbé condenser, using the concave mirror and partially closed diaphragm.

2. Repeat this procedure, using a drop of the broth culture of *Bacillus proteus mirabilis*. Slight motility of the bacilli may be seen.

3. Examine in the same manner a drop of putrefying urine (48 hours old). Large numbers of bacteria, usually of several species, are seen.

Hanging Drops.

Hanging drop preparations are made to study motility of bacteria. Under the microscope we may make out three kinds of movement: (a) Current movement, where the bacteria move with the suspending fluid. (b) Molecular or "Brownian" movement, where the bacteria vibrate in the fluid, as do all solid particles suspended in fluids. In this movement there is no actual change in position of the bacterial cells. (c)

Actual movement, where the bacteria swim or move about in the suspending fluid. This last is what we mean by motility of a bacterium.

4. Make a hanging drop preparation from the 18 hours, broth culture of *Bacillus typhosus* supplied.

(a) Transfer a drop of the culture to a clean cover glass. (Never neglect aseptic precautions).

(b) Ring about with vaseline, the depression on a hollow ground slide.

(c) Invert the cover glass over the cell and press firmly into the ring of vaseline, so as to exclude the air.

In examining the preparation find the edge of the drop with the low dry power, then fixing the slide, examine with the oil-immersion lens, partially closing the diaphragm.

5. In like manner examine the liquefied gelatine culture of *Staphylococcus pyogenes aureus*.

No motility is present, but Brownian movement can be noted.

Hanging Drop Cultures.

Hanging drop cultures are used to study the manner of division, the process of spore formation and germination, and the motility of bacteria. The three former require continual microscopic observation of the culture drop, at certain periods.

6. Make hanging drop cultures of *Bacillus anthracis* and of *Streptococcus pyogenes*.

(a) Place cleansed cover glasses, sterilized by passing through the flame, on a strip of wire gauze, sterilized

in like manner, and covered by a bell jar, sterilized by washing in 1:1000 bichloride of mercury.

(b) With a sterile loop carefully place drops of broth (sterile) on the cover glasses.

(c) Lightly inoculate these drops with the respective bacteria.

(d) Invert these drops over the cell of a hollow ground slide, previously sterilized, and having edge of cell ringed with vaseline.

(e) Place in the incubator at 37°C, on slide rack.

DEMONSTRATION V.

Examination of Hanging Drop Cultures—Spores and Spore Staining.

1. Examine the hanging drop culture of *Streptococcus pyogenes* made last day, with $\frac{1}{2}$ oil-immersion lens; at the margin of the drop long chains of streptococci can be seen.

2. Stain the hanging drop culture of *Streptococcus pyogenes*, just examined.

(a) Remove the oil carefully with blotting paper, and then raising up the cover glass, clear away the vaseline from its edges.

(b) Dry, and pass film three times through the flame.

(c) Stain the film by Gram's method.

3. Examine the hanging drop culture of *Bacillus anthracis* with the low and high dry lenses and the $\frac{1}{2}$ oil immersion.

Note the long segmented threads at margin of culture drop, and the presence in the rods of oval refracting bodies—spores.

4. Stain the hanging drop culture of *Bacillus anthracis* with Loeffler's methylene blue, removing the cover glass as described above, and clearing the film before staining in 10% acetic acid.

The spores remain unstained as oval refracting bodies, both free and in the rods.

Staining of Spores.

Potato cultures of *Bacillus anthracis* and *Bacillus lactis viscosus* are supplied. (*B. megatherium*, *B. subtilis* or *B. filamentosus* might be employed.)

(a) Prepare thin cover glass films of these bacteria in the usual manner, dry and fix in the flame.

(b) Float in Ziehl's carbol-fuchsin, or in anilin water fuchsin, for 20 to 30 minutes, keeping the staining fluid warm.

(c) Wash the films in water and rinse for two to three seconds in acid alcohol. (Alcohol 97 cc., Hydrochloric acid 3 cc.) At this stage the specimen may be examined by mounting in a drop of water. The acid decolorizes the rods, leaving the spores stained. If the bodies of the bacilli are still red, the film must be again washed through the acid alcohol. If the spores are not stained place the film again in the carbol-fuchsin.

(d) If the spores are properly stained, place the film for one to two minutes in Loeffler's methylene blue to stain the rods.

(e) Wash in water, dry thoroughly and mount in Canada balsam.

The spores are stained red, rods blue.

DEMONSTRATION VI.

Staining of Flagella—Cultures of *Bacillus Anthracis***Flagella Staining.**

Ordinary methods of staining do not suffice to demonstrate flagella, so that special staining processes are required. Emulsions in sterile distilled water of 18 hour agar cultures of *Bacillus typhosus* and of *Spirillum cholerae asiaticae* are furnished.

1. Muir's modification of Pitfield's method.

The following re-agents are required:

The mordant,

10% aqueous solution tannic acid, filtered	10 cc.
Saturated aqueous solution, bichloride of mercury	5 cc.
Saturated aqueous solution, alum	5 cc.
Ziehl's carbol-fuchsin	2 cc.

This mordant will keep for one or two weeks.

The stain,

Saturated aqueous solution of alum, filtered	10 cc.
Saturated alcoholic solution of gentian violet	2 cc.

This stain will keep two or three days.

The cover glasses used must be thoroughly cleansed. This may be easily effected by rubbing first with a clean cloth, then washing in a mixture of equal parts of

alcohol and ether, and while moist passing them through the flame. Cover glasses may also be cleansed by the method advised by van Ermenghem, as follows:

Boil the cover glasses for five minutes in the following solution,

Potassium bichromate	6 grammes.
Sulphuric acid	6 grammes.
Water	100 cc.

From this solution wash through several dishes of distilled water, and then place in alcohol. Before using pass through the flame.

(a) Prepare thin films from the emulsions; dry and fix in the flame.

(b) Cover the film (held in forceps) with the mordant, and hold high over the flame till it steams gently for one minute. (Or float the film in the warmed mordant for the same period.)

(c) Wash thoroughly in large amounts of distilled water.

(d) Pour on now some of the stain, and again heat gently for one minute (or place in the warmed stain).

(e) Wash in water, dry and mount in Canada balsam.

Those bacteria which stain by Gram's method can be treated after the stain by iodine in the usual manner. Fair results are usually obtained by this method. Better microscopic pictures can be secured by van Ermenghem's method, but his method is longer and usually requires considerable practice.

Cultures of Bacillus Anthracis.

From the culture tube supplied inoculate,

- (a) an agar-agar (slope) tube;
- (b) a potato tube;
- (c) a gelatine (stab) tube;
- (d) a broth tube;
- (e) a broth tube, to which carbolic acid has been added in proportion of 1 to 1000;
- (f) a litmus milk tube.

Place all except (c) in the incubator at 37°C, and keep (c) at 20°C.

Examine these growths at the end of 24, 48 and 72 hours, noting the characters of growth on the various media.

DEMONSTRATION VII.

Bacillus Anthracis in pure cultures—Tissues of animal dead of Anthrax Septicaemia—Inoculation of animal with Bacillus Anthracis.

1. Make cover glass preparations from the agar, and the broth cultures of *Bacillus anthracis*.

Stain with Loeffler's methylene blue, diluted gentian violet or by Gram's method.

2. Make cover glass preparations from the carbolized broth culture of *Bacillus anthracis*.

Stain with Loeffler's methylene blue. No spores are seen.

3. Inoculate two litmus milk tubes, one from the ordinary broth culture and the second from the carbolized broth culture of *Bacillus anthracis*.

Incubate at 37°C and examine day by day. The latter produces less acid. (By animal experiments it could also be shown to be less virulent).

Tissues of Animal dead of Anthrax Septicaemia.

Sections from lung, liver and kidney are supplied.

4. Staining with Loeffler's methylene blue.

(a) Place sections in the stain for 5 to 10 minutes.

(b) Remove excess of stain by washing in water.

(c) Place in $\frac{1}{2}$ to 1% acetic acid for 10 to 20 seconds, till the sections become a light blue.

- (d) Immediately wash in fresh water.
- (e) Dehydrate in absolute alcohol for 1 to 2 minutes.
- (f) Clear in xylol for 1 to 2 minutes.
- (g) Transfer to slide with section lifter and mount in Canada balsam.

Sections after dehydration may be transferred to slide and cleared there with xylol before mounting, as xylol causes at times considerable shrinkage and curling.

Examine with the low and high dry lenses and the $\frac{1}{2}$ oil immersion. Anthrax bacilli are seen in the capillaries of the organs.

5. Staining by Gram's method and eosin.

- (a) Place the sections in alcohol for 1 minute.
- (b) Transfer to anilin gentian violet, 5 to 10 minutes.
- (c) Rinse the sections in distilled water, and then place for 2 minutes in Gram's iodine solution.
- (d) Rinse in water, and then in alcohol for $\frac{1}{2}$ minute.
- (e) Place for 1 minute in eosin staining fluid,

Alcoholic eosin5 gramme.
Alcohol	.	.	.	70 cc.
Water	.	.	.	30 cc.
- (f) Remove excess of eosin by washing in water and then in alcohol.
- (g) Transfer to acid alcohol for 5 to 10 seconds, and again wash in alcohol till the sections become red.
- (h) Clear in xylol, transfer to slide, and mount in Canada balsam.

6. Staining with picocarmine and Gram's method.

(a) Place the sections in picocarmine 20 to 30 minutes.

(b) Wash in water to which a few drops of hydrochloric acid have been added.

Then pass the sections through the process described under (5) with the exception of counterstaining with eosin.

Inoculation of Mice or Guinea Pigs Subcutaneously with Bacillus Anthracis. (Class Demonstration.)

The animal is placed in a holder or held by an assistant. The hair is clipped from the point of inoculation, (root of tail in mouse, inner side of thigh in guinea pig). The skin at this point is then washed with 1:1000 solution of bichloride of mercury. With a sterile scissors a small snip is made through the skin. With a platinum loop a drop of 48 hours broth culture of Bacillus anthracis is introduced and pushed well up under the skin. The point of introduction is then lightly seared with a heated glass rod.

DEMONSTRATION VIII.

Examination of guinea pig dead of Anthrax Septicaemia—Plate cultivations—Cultures of the Pyogenic Bacteria.

Guinea Pig dead of Anthrax.

1. (a) Carefully examine the animal, noting the swelling extending from the groin, to a variable extent over the abdomen. Then stretch out the animal by tacking down the legs to a board, moistened with 1:1000 bichloride of mercury solution.

(b) Lay the hair of the animal by moistening with methylated spirit.

(c) With a sterilized scissors carefully cut through the skin along the median line and reflect it from over the thorax, abdomen and thighs.

(d) Note the situation and character of the œdema. From the œdema fluid, smear cover glass films for after staining. (Cultures might also be made.)

(e) Sterilize the abdominal wall in the midline and the thorax at the rib-costal junctions, with a flat-bladed knife, heated to redness.

(f) With sterilized forceps and scissors open into the abdomen along the midline. With fresh forceps remove the spleen and break a portion of it up in a sterile capsule. From the broken pulp, make cover glass preparations by placing a drop of pulp on one cover glass and dropping a second cover glass upon this,

spread out the drop into a thin film, and carefully slide the two cover glasses apart.

(*g*) Open carefully the thorax, cutting through the costal cartilages, and expose the heart.

(*h*) Sterilize a portion of the exposed heart wall by searing it with a heated glass rod. Push through this part, a sterile pipette and withdraw the blood of the chambers. Several pipettes may be filled in this manner, from one of which cover glass preparations can be made as described in (*f*) for staining. On withdrawing pipettes fuse the open ends carefully in the flame. Preserve the pipettes for the making of plate cultivations.

The remaining viscera of the animal may be removed and preserved in 4% formalin for the after study of sections.

Staining of the Cover Glass Films.

Fix the films after drying by passing through the flame. Better results are secured in staining the preparations from the spleen pulp and the blood by fixing the films in a 10% solution of formalin in 90% alcohol, for one minute.

2. Staining with eosin and methylene blue.

- (*a*) Place the fixed films in eosin $\frac{1}{2}$ to 1 minute.
- (*b*) Wash in water and then dry.
- (*c*) Place now in Loeffler's methylene blue for 1 to 2 minutes.
- (*d*) Wash in water, dry and mount in Canada balsam.

Examine with the dry and oil-immersion lenses. Anthrax bacilli and nuclei are stained blue, the red corpuscles and eosinophile granules of leucocytes, pink.

Spores are not seen in the animal body.

3. Staining by Gram's method and eosin.

(a) If films have been fixed by heat place in alcohol first for 1 minute; if by formalin place at once in anilin gentian violet for 2 to 3 minutes.

(b) Rinse in water and transfer to Gram's iodine for 1 minute.

(c) Rinse again in water and then in alcohol till stain ceases to come away.

(d) Place in eosin, $\frac{1}{2}$ to 1 minute.

(e) Wash in water, dry and mount in Canada balsam.

Plate Cultures.

4. Make plate cultivations from blood of heart of guinea pig, dead of Anthrax septicaemia.

(a) Liquefy two or three tubes of gelatine in water bath, or incubator at 37°C.

(b) Observing all aseptic precautions, with a sterilized forceps break off the fused end of pipette containing blood from the heart, and by carefully applying heat to bulb of pipette, allow one drop of blood to fall into one of the liquefied gelatine tubes.

Label this tube No. 1.

(c) From tube No. 1, after careful admixture of the blood drop, transfer three loops to a second liquefied tube (No. 2). If microscopic examination has shown a

large number of bacilli to be present, a further dilution can be made from tube No. 2.

(*d*) Heat the tops of the inoculated tubes to assure thorough sterilization, and then permit them to cool.

(*e*) Remove the plugs and carefully pour the gelatine into sterilized Petri dishes, causing the gelatine to spread thoroughly over the plate surface.

(*f*) Set the gelatine by placing the plates on blotting paper moistened with cold water, and then place in incubator at 20°C.

Examine at the end of 24, 48 and 72 hours, noting the character and number of colonies, and the presence of other species of bacteria.

Esmarch roll tubes may be prepared instead of plates, by spreading the liquefied gelatine over the sides of the tubes and setting the gelatine rapidly by revolving the tubes on ice.

5. Make cultures of *Staphylococcus pyogenes aureus*, *Staphylococcus pyogenes albus* and *Bacillus pyocyaneus* in agar, potato and gelatine (stab) tubes.

6. Make cultures of *Streptococcus pyogenes* in agar, gelatine (slope) and broth tubes.

Examine these cultures day by day, noting the general characters of growth.

DEMONSTRATION IX.

Impression Specimens of *Bacillus Anthracis*—Pyogenic Micrococci—
Staining of Pus—Agar Plates.

1. Impression specimens of *Bacillus anthracis*.

(a) Examine the Anthrax plate cultures and when colonies appear, gently press over a part showing them a sterile cover glass.

(b) Carefully raise the cover glass, dry by holding high over the flame, and pass three times through the flame.

(c) Stain the film in Loeffler's methylene blue, or by Gram's method.

A stained impression of the colonies will be obtained.

2. Prepare cover glass films from the agar or potato cultures of *Staphylococcus pyogenes aureus, albus, and Bacillus pyocyaneus*.

Stain with anilin gentian violet, or Gram's method. (*B. pyocyaneus* does not stain by Gram's method.)

3. Prepare cover glass films from the agar and the broth cultures of *Streptococcus pyogenes*.

Stain with anilin gentian violet, or by Gram's method. Contrast the appearances microscopically.

4. Staining of pus from an abscess.

(a) Make cover glass films by placing a drop of the pus (obtained after the first gush, from an opened

abscess) on a cover glass, dropping upon this a second cover glass and sliding the two apart.

(b) Dry and fix by passing through the flame.

(c) Stain these films,

(1) with eosin and methylene blue (page 21);

(2) with diluted gentian violet;

(3) by Gram's method and eosin (page 18).

Agar Plates from Pus.

5. (a) Liquefy two agar tubes by heating to 100°C. for several minutes. Permit these tubes to cool in water. (Agar sets at 38°C.)

(b) Inoculate a few cc. of sterile water or a broth tube with two loops of the pus furnished.

(c) When the agar tubes have cooled to 42°C. rapidly inoculate a tube with three loops from the inoculated water or broth tube.

(d) From this tube inoculate the second tube, using three loops. Place the tubes in water at 42°C.

Sterilize the tops of the tubes, and when cooled pour into Petri dishes in the same manner as described in making gelatine plates.

Incubate at 37°C., examining after 24 and 48 hours.

The use of plates in ordinary practice is not necessary, as tubes can be smeared either directly from the pus, using minute amounts, or better, can be smeared from the dilution in sterile water or broth.

DEMONSTRATION X.

Examination of Plate cultures—Gonorrhœal pus—Pus from Actinomycosis.

1. Examine the plate cultures from pus, made last day. Make agar and gelatine sub-cultures of any different varieties of bacteria that may be present.

Examine the cultures daily, noting characters of growth, so as to aid identity of the species.

2. Make cover glass preparations from any different varieties of bacteria developing on plates.

Stain with anilin gentian violet, and also by Gram's method.

Staining of Gonorrhœal Pus.

In the usual manner prepare cover glass films of the pus. It is best to make these directly from the discharges.

3. Stain the dried and fixed films in eosin and methylene blue.

Note the relationship of the Gonococci to the pus cells.

4. Stain other films in anilin gentian violet for one minute. Mount these films in water and examine for Gonococci. If present, remove film from slide and wash rapidly several times through alcohol. Then wash in water, dry and mount in the usual manner. The Gonococcus is decolorized. A second film may be treated by Gram's method. The Gonococcus does not retain the stain, when so treated.

Pus from Actinomycosis.

Note the minute greyish yellow granules in the pus.

5. Place on a slide a drop of the pus, containing one or more granules. Treat it with several drops 10% caustic potash solution and press on a cover glass.

Examine with low and high dry lenses. Radiating masses of the fungus and separate threads can be readily detected.

6. Prepare cover glass films from the pus, and stain by Weigert's modification of Gram's method.

(a) Place the films in alcohol 1 to 2 minutes.

(b) Transfer to anilin gentian violet, 10 to 15 minutes.

(c) Rinse in water and then transfer for 2 to 3 minutes to Weigert's iodine solution,

Iodine	1 gramme.
Potassium iodide	2 grammes.
Water	100 cc.

(d) Rinse again in water. Counterstain in eosin for 1 minute.

(e) Wash in water and remove excess of water with blotting paper.

(f) Dehydrate and decolorize in a solution of anilin oil 2 parts, xylol 1 part, till violet color has almost disappeared.

(g) Wash in xylol to remove all anilin oil and then mount in Canada balsam.

Examine with the low and high dry lenses and with the oil-immersion lens.

DEMONSTRATION XI.

**Micrococcus Lanceolatus (Pneumococcus) in Sputum—Bacillus Influenzæ in Sputum—Sections of Pneumonic Lung—
Tissues containing Pyogenic Micrococci.**

Pneumonic Sputum.

Fresh sputum is furnished when possible, otherwise sputum preserved in 5% carbolic acid solution is used. Films prepared from the preserved sputum have to be cleared, after fixation in the flame, by placing in a mixture of equal parts alcohol and ether for 1 to 2 minutes.

Prepare cover glass films from the sputum by placing a small portion on a cover glass and, after pressing on a second cover glass, sliding the two apart.

1. Stain the films so prepared, by Gram's method, counterstaining with eosin.

This method stains the capsule fairly well.

2. Stain other prepared films of the pneumonic sputum in eosin and methylene blue.

3. For staining the Pneumococcus and its capsule in fresh sputum, use the following method:

(a) Prepare films in the usual manner, dry and fix in the flame.

(b) Pass the films quickly through glacial acetic acid.

(c) Without washing, place the film in anilin gentian violet for 5 to 6 minutes.

(d) Rinse in water, dry and mount in Canada balsam.

4. Prepare cover glass films from the sputum, (preserved in 5% carbolic acid), of a patient with Influenza Bronchitis. Dry and pass through the flame. Clear for 1 to 2 minutes in the alcohol-ether mixture.

Stain in Loeffler's methylene blue or in anilin gentian violet in the usual manner.

The Influenza bacilli appear as fine short rods, at times forming fine segmented threads. Many lie in pus cells.

**Staining of Pneumonic Lung (red hepatization),
for Fibrin and Pneumococci.**

5. Owing to the fragile nature of the sections, the staining processes are best carried out on a slide. The use of such fixing fluids as the glycerin albumin mixture, should be avoided, owing to the precipitation of the stains. The sections are given out in alcohol.

(a) With a section lifter remove a section to a slide, and remove excess of alcohol with blotting paper.

(b) Cover the specimen with anilin gentian violet for 15 minutes, protecting from dust by placing under a bell jar.

(c) Remove the stain by blotting paper and carefully wash with water.

(d) Treat the sections with Weigert's iodine for 2 to 3 minutes.

(e) Remove the iodine solution and wash in water.

(f) Cover the section with eosin for 1 minute, and then wash off with water.

(g) Remove the water with blotting paper, and treat with the anilin oil—xylol mixture, till no more blue stain comes away.

(h) Wash several times in xylol and mount in Canada balsam.

The section might be previously stained in picrocarmine, and then put through this process, with the exception of the eosin staining. Either method usually shows both the fibrin net work and Pneumococci well, when examined with the $\frac{1}{12}$ oil immersion lens.

Staining of Tissues for Pyogenic Micrococci.

6. Stain sections of erysipelas of rabbit's ear.

(a) By Gram's method, counterstaining in eosin (page 18).

(b) By Gram's method, previously staining in picrocarmine (page 13).

7. Stain the sections of kidney, from acute suppurative nephritis. (*Staphylococcus pyogenes aureus*).

(a) With eosin and methylene blue (page 21).

(b) By Gram's method, previously staining with picrocarmine or counterstaining in eosin.

These methods may be employed for other tissues, containing the pyogenic micro-organisms.

DEMONSTRATION XII.

Pure Cultures of Tubercle Bacillus—Tubercular Sputum.

1. From the glycerine agar cultures of the Tubercle bacillus prepare cover glass preparations, and stain in the following manner :

(a) Float in Ziehl's carbol fuchsin for 5 minutes.

(b) Wash in water and then rinse quickly through 33½% nitric acid solution.

(c) Wash immediately in 70 % alcohol and then in water.

(d) Dry and mount in Canada balsam.

2. Prepare other cover glass films and stain in sudan III. (Dorset).

Sudan III staining fluid consists of a saturated solution of sudan III in 80% alcohol.

(a) Place the prepared films in the stain for 5 minutes.

(b) Wash the films in 70% alcohol and then in water, dry and mount in Canada balsam.

Sudan III has a selective affinity for fat, which is present in large amount in the bodies of Tubercle bacilli.

Examination of Sputum for Tubercle Bacilli.

Pour a thin layer of sputum into a glass capsule and pick out for examination a yellowish (caseous) particle if present; otherwise choose a purulent part.

Prepare cover glass films by pressing out the particles between two cover glasses, drying and fixing in the flame.

• 3. Staining by Ziehl-Neelsen's method.

(a) Float the films in Ziehl's carbol-fuchsin for 5 to 10 minutes, keeping the stain warm. (Do not boil).

(b) Wash off the excess of stain in water.

(c) Rinse quickly through 33 $\frac{1}{3}$ % nitric acid. The red color disappears at once.

(d) Wash immediately in 70% alcohol for 1 to 2 minutes. If the red color which re-appears is persistent, again pass the film into the acid and rinse in the alcohol.

(e) Wash the film in water and dry.

(f) Counterstain in Loeffler's methylene blue for $\frac{1}{2}$ to 1 minute. Wash in water, dry and mount as usual.

Tubercle bacilli appear as red rods; other bacteria and the nuclei of cells are stained blue.

This method is decidedly the most accurate for routine examination work.

4. Staining by Gabbett's method.

The same method of procedure is followed till after the removal of the film from the carbol-fuchsin. It is then placed for 30 seconds in Gabbett's solution:

Methylene blue	.	.	.	2 grammes.
Sulphuric acid	.	.	.	25 cc.
Water	.	.	.	75 cc.

The film is then washed in water, dried and mounted in the usual manner.

This method is shorter but it has not proven so accurate, nor does it give as clear a film as the Ziehl-Neelsen process.

5. Staining in sudan III.

(a) Place the prepared films in sudan III for 5 minutes.

(b) Wash for 5 minutes in 70% alcohol.

(c) Wash in water and then dry.

(d) Counterstain for 1 minute in Loeffler's methylene blue. Wash in water, dry and mount in the usual manner.

**Examination of Sputum or Fluids containing
but few Tubercle Bacilli.**

When Tubercle bacilli are suspected but cannot be demonstrated by ordinary examinations, several methods may be adopted for further microscopic investigation.

(a) Van Ketel's method. Add to the sputum about three or four times its volume of 5% carbolic acid. If the sputum is watery, add pure carbolic acid to make a 5% solution. This is particularly applicable when examining urine. Shake the mixture thoroughly, for at least five minutes, to break up sputum particles. Pour into a conical urine glass and allow to stand 12 to 24 hours. With a pipette, withdraw the lowest portion of sediment for examination by any of the methods already given. Films must be cleared in the alcohol-ether mixture, before staining.

(b) A more rapid method is to add to the sputum sufficient 10% caustic potash solution, to liquefy it.

This liquefied fluid is allowed to stand for 12 to 24 hours in a conical urine glass, and the sediment examined. Or a portion of the liquefied sputum may be centrifuged and the sediment examined at once by the usual methods. In urine, smegma bacilli give staining re-actions allied to those of the bacillus of Tuberculosis. If the Ziehl-Neelsen process is accurately carried through, only rarely will there be danger of mistaking smegma bacilli for Tubercle bacilli. Sudan III is however, a positive differential stain.

When microscopic methods fail, inoculation of a guinea pig subcutaneously or intraperitoneally, will lead to the development of Tuberculosis, if any living bacilli are inoculated.

DEMONSTRATION XIII.

Sections of Tubercular Tissues—Leprosy Tissues—Cultures of
Tubercle Bacillus from an Inoculated Animal—
Cultures of Bacillus of Diphtheria.

Tubercle Bacilli in Tissues.

Sections are furnished (in water), from acute miliary Tuberculosis of lung, and also of Tuberculosis of lung of cattle.

1. Staining in fuchsin solutions.

(a) Place the sections in carbol-fuchsin or in anilin fuchsin for from 30 minutes to 24 hours, keeping the stain covered in the incubator at 37°C. Better results are obtained by leaving in stain for 24 hours.

(b) Rinse the sections in water, and then transfer for a few seconds to 33½% nitric acid.

(c) Wash at once in 70% alcohol till no more red stain comes away. If the section is still red, wash again through the acid and then in the alcohol.

(d) Rinse in water and place in Loeffler's methylene blue for 1 minute.

(e) Rinse again in water, dehydrate in absolute alcohol, clear in xylol and mount on slide in usual manner.

Sections from paraffin blocks, received into water at 48°C, may be fixed on cover glasses and stained as cover glass films in the following manner:

Float a section on a cover-glass and removing from water, blot firmly and evenly with several layers of

blotting paper. Heat the section carefully to melt the paraffin, rinse through xylol to free the section from the paraffin, then in alcohol to free from xylol. From the alcohol place directly into the carbol-fuchsin.

2. Staining in sudan III solution.

(a) Place the sections for 10 minutes in the sudan III stain.

(b) Wash for 5 minutes in 70% alcohol.

(c) Rinse in water and transfer for 1 minute to Leoffler's methylene blue.

(d) Rinse in water, dehydrate in absolute alcohol, clear in xylol and mount in Canada balsam.

Leprosy Tissues.

Bacillus leprae stains like the Tubercle bacillus, but more readily, and is more rapidly decolorized. It does not stain with sudan III.

3. Stain sections by the following methods:

(a) Carbol-fuchsin for 20 to 30 minutes, treating afterwards as for tuberculous tissues.

(b) Weigert's method and eosin.

The Leprosy bacilli are always present in large numbers in the leprous nodules, and the discharges in ulcerating tubercular leprosy.

Cultures of Tubercle Bacilli from Guinea Pig.

The guinea pig has been inoculated three or four weeks previously with sputum from pulmonary Tuberculosis, and has been killed by chloroforming.

The animal is opened in the usual manner, and the enlarged inguinal and iliac glands are removed with a sterile forceps, and broken up in sterile capsules.

4. From the pulp in the capsules inoculate freely,

- (a) 6 blood serum tubes;
- (b) 6 glycerine broth tubes;
- (c) 6 glycerine agar tubes.

After inoculation seal the tubes with ordinary wax or paraffin, so as to prevent evaporation.

Incubate at 37°C. and examine at the end of 7, 10, 14, and 21 days.

5. From the culture of *Bacillus diphtheriæ* furnished inoculate,

- (a) a blood serum tube;
- (b) a glycerine agar tube (slope);
- (c) a broth tube.

Place in the incubator at 37°C. and examine at end of 18, 24 and 48 hours.

DEMONSTRATION XIV.

Diphtheria Bacillus—Cultures of Spirilla of Asiatic Cholera, of Metchnikof, and of Finkler and Prior—Inoculation of Animals with Diphtheria Bacilli—Diphtheria Toxin—Antitoxin.

1. Make cover glass preparations from the colonies of *Bacillus diphtheriæ* on the blood serum and glycerine agar tubes.

Stain with Loeffler's methylene blue, and also by Gram's method. Note the variable morphology of the bacilli, and the differences between the growth on the different culture media.

Clinically, cultures are made on blood serum or glycerine agar, from suspected throats, and after incubation for 16 to 20 hours, the tubes are examined. The material is best removed from the throat by a small swab, prepared by wrapping a fragment of absorbent cotton about the end of a fairly rigid wire, which is then placed in a plugged test tube and sterilized in the hot air sterilizer. The swab is placed against the membrane, and firmly pressed upon it with a slight twirling motion, taking care not to touch other parts. The swab is then lightly rubbed over one or more culture tubes. Immediate staining and microscopic examination might be made of the material on the swab, but owing to the presence of numerous saphrophytic bacteria, and of bacteria which somewhat closely resemble *Diphtheria* bacilli in morphology, it is best to depend on cultural examination.

2. Make inoculations of *Spirillum cholerae asiaticæ*, *Spirillum Metchnikovi*, and *Spirillum* of Finkler and Prior on,

- (a) agar tubes (slope);
- (b) gelatine tubes (stab);
- (c) peptone tubes.

Toxins and Antitoxins. (Class Demonstration).

(a) A medium sized guinea pig (about 400 grammes) is inoculated subcutaneously with .5 cc. of a 24 hours broth culture *Bacillus diphtheriæ* (virulent). The animal usually dies in from 24 to 36 hours.

Broth cultures of *Bacillus diphtheriæ* are filtered through porcelain (Chamberland's candles), to obtain the toxins free from the bacilli. Guinea pigs are inoculated and the maximum fatal dose learned and the toxin is then standardized. (.01 cc. of standard toxin kills a medium sized pig in $3\frac{1}{2}$ days).

(b) A guinea pig is inoculated with the minimum dose of the toxin (death in 3 days).

(c) A guinea pig is inoculated with 100 times the minimum fatal dose, plus one unit of some standard antitoxin. The animal recovers.

(d) A guinea pig is inoculated with 100 times the minimum fatal dose. 15 minutes afterward one unit of antitoxin is inoculated. The animal dies.

DEMONSTRATION XV.

Spirillum of Asiatic Cholera—Spirillum of Finkler and Prior—Spirillum Metchnikovi—Indol Test—Diphtheria Membrane—
Intraperitoneal Inoculation of Animals.

1. Examine hanging drop preparations from the peptone cultures, of each of the spirilla.

2. Make cover glass preparations from the agar and the peptone cultures, of each of the spirilla.

Stain with anilin fuchsin for 30 seconds and mount in the usual manner.

Contrast the preparations from the different culture media.

3. Make and stain cover glass preparations from the 10 days old, potato cultures of the Cholera spirillum.

Many involution forms will be found.

4. To the peptone cultures of each of the spirilla add carefully, with a pipette, 10 drops of sulphuric acid.

A rose red coloration appears with *Spirillum cholerae asiaticae* and *Spirillum Metchnikovi*, but not with the *Spirillum* of Finkler and Prior. This is due to the formation by the two former of indol and nitrites. Indol only gives this reaction in the presence of nitrites. If no reaction follows the addition of the acid, add 1 cc. of a .01% solution of potassium nitrite, freshly prepared. If the reaction does not then develop, indol is certainly absent.

Stain the sections of diphtheria membrane furnished.

(a) By Gram's or Weigert's method, counterstaining with eosin.

Carry through the staining processes on the slide.

(b) In Czinzinski's solution,

Alcoholic eosin—.5% solution in 70% alcohol	20 cc.
Saturated aqueous solution, methylene blue	40 cc.
Water	40 cc.

Place the sections in this stain for 6 to 24 hours. Wash thoroughly in water till all blue color is removed, that will come away. Dehydrate in absolute alcohol, clear in xylol and mount in usual manner.

Intraperitoneal Inoculation of Guinea Pigs.

(Class Demonstration.)

A medium sized guinea pig is chosen. The hair is removed from the median line of abdomen and the skin is washed with 1 in 1000 bichloride of mercury solution. With a sterilized hypodermic syringe .5 cc. of a 24 hour broth culture of moderately virulent *Spirillum cholerae asiaticae* is thrown into abdomen, the needle being entered about the centre of the mid-line. The animal usually dies in from 18 to 36 hours.

DEMONSTRATION XVI.

Examination of animal inoculated with *Spirillum Cholerae Asiaticae*—
Cultures of *Bacillus Typhosus* and *Bacillus Coli*
Communis—Typhoid Tissues.

**Examination of animal inoculated with the *Spirillum*
of Cholera.**

Open up the abdominal cavity of the guinea pig, using all aseptic precautions (page 20).

Note the condition of the peritoneum and character of the exudate.

1. With a sterile loop, or capillary pipette, transfer a drop of the peritoneal exudate to a liquefied gelatine tube. From this tube inoculate a second.

Pour plates in the usual manner.

Incubate at 20°C. and examine carefully on successive days.

2. With a sterilized loop, smear a series of cover glasses, with the peritoneal exudate.

Stain some films with eosin and methylene blue; others may be stained with anilin gentian violet or carbol fuchsin after clearing in 10% acetic acid.

carefully open the thorax, sterilize a portion of exposed heart surface, and with a capillary pipette, withdraw blood from the chambers.

From this blood make cover glass preparations, fix in formalin solution, and stain with eosin and methylene blue.

4. Make inoculations of *Bacillus typhosus* and *Bacillus coli communis* on,

- (a) agar-agar tubes;
- (b) gelatine (slope) tubes;
- (c) liquefied grape sugar gelatine;
- (d) potato tubes;
- (e) peptone tubes;
- (f) litmus milk tubes.

Incubate all except the gelatine tubes at 37°C. Examine and contrast the growths from day to day.

5. Sections of mesenteric gland, spleen and liver from Typhoid fever cadaver.

In the tissues *Bacillus typhosus* is found scattered in clumps, so that a number of sections may have to be examined before finding any bacilli. At least six sections of each of the tissues should be stained.

(a) Stain in Loeffler's methylene blue, leaving in the stain from 30 minutes to 2 hours. Rinse in water and then in .1% acetic acid for 2 or 3 minutes; dehydrate in absolute alcohol, clear in xylol and mount in balsam.

- (b) Stain in Stirling's gentian violet solution,
- | | |
|--------------------------|------------|
| Gentian violet | 5 grammes. |
| Alcohol | 10 cc. |
| Anilin oil | 2 cc. |
| Water | 88 cc. |

Leave sections in this stain for 10 minutes. Rinse in water and then in .1% acetic acid for 2 or 3 minutes; dehydrate in alcohol, clear first with oil of cloves and then wash several times with xylol before mounting in balsam.

DEMONSTRATION XVII.

Cultures of *Bacillus Typhosus* and *Bacillus Coli Communis*—Serum
Diagnosis of Typhoid Fever—Cultures of *Bacillus Pestis*
Bubonicæ, *Bacillus Icteroides* and
Bacillus Mallei.

1. Make and compare hanging drop preparations from the peptone tubes of *Bacillus typhosus* and *Bacillus coli communis*.

2. Examine the peptone cultures of *Bacillus typhosus* and *Bacillus coli communis* for indol, adding to each tube 1 cc. of a .01% fresh solution of potassium nitrite, before the addition of the sulphuric acid.

Bacillus coli communis gives the indol reaction.

3. Make cover glass preparations of *Bacillus typhosus* and *Bacillus coli communis*. Stain in anilin gentian violet or in Loeffler's methylene blue.

The serum diagnosis of Typhoid Fever and Typhoid Bacilli.

In the blood serum of animals immunized against *Bacillus typhosus*, and also, at an earlier or later date, in the blood of persons with Typhoid fever, there are present certain constituents which possess the specific property of agglutinating liquid cultures of Typhoid bacilli. The phenomena noted microscopically are loss of motility of the bacilli and their aggregation in clumps. In tube experiments, we have precipitation of the contained bacilli. For the proper application of

this test, an 18 to 24 hour broth culture, or suspension, of moderately virulent Typhoid bacilli, and certain dilutions of the serum used, are required.

A dilution of at least 1 part of serum in 30 is necessary. The time limit for the reaction should be set at 2 hours. Usually the phenomena are noted within a few minutes.

Blood, from a fatal case of Typhoid fever, dried on sterile thin filter sheets and cut into squares of 1 cc. is furnished. (Blood may also be preserved in standardized capillary pipettes).

4. With sterile forceps place a dried blood square in a sterile glass capsule. With a medicine dropper carefully add 15 drops of sterile distilled water, so as to extract the agglutinating constituents.

On a sterile cover glass place one drop of this extract, and add one drop of the 18 hour broth culture of *Bacillus typhosus* furnished. This makes a dilution of about 1 in 30.

Examine in hanging drop, under the microscope, and note the characteristic reaction.

5. Repeat this procedure, using 1 drop of the extract to 3 drops of the broth culture of *Bacillus typhosus*, giving a dilution of about 1 in 60. Examine as before and note results.

6. Repeat this procedure using 1 drop of the extract, and 1 drop of a broth culture of *Bacillus coli communis*. No clumping occurs.

For clinical examination in cases of Typhoid fever, blood may be taken from the lobe of the ear or the fin-

ger, after thorough cleansing of the parts, and allowed to dry on firm white paper or on a slide. The above method can then be carried out.

Cabot advises an excellent method for bedside examination. With a medicine dropper, one drop of blood from the patient is added to 30 (or more) drops of broth culture, or emulsion of Typhoid bacilli. Drops from this dilution are then examined in hanging drop preparations.

7. Make inoculations of *Bacillus pestis bubonicæ* and *Bacillus icteroides* on,

- (a) agar tubes;
- (b) gelatine (slope) tubes;
- (c) broth tubes.

Inoculate with *Bacillus mallei*,

- (a) an agar tube;
- (b) a potato tube.

Examine these tubes from day to day, noting characters of growth.

DEMONSTRATION XVIII.

Bacillus Pestis Bubonicæ—Bacillus Icteroides—Bacillus Mallei—
Cultures of Tricophyton Tonsurans and Achorion
Schonleinii—Hair in Tinea Tonsurans.

1. Prepare cover glass films from the agar cultures of *Bacillus pestis bubonicæ*, *Bacillus icteroides* and *Bacillus mallei*. Stain in anilin gentian violet or in anilin fuchsin.

2. Remove in the usual manner a small portion of the growth of *Tricophyton tonsurans* from the cultures furnished. Tease out on a slide after treatment with 10% caustic potash solution. Examine with the low and high dry lenses.

3. In like manner examine a preparation from the culture of *Achorion Schonleinii* (Favus).

Compare this fungus with *Tricophyton tonsurans*.

4. Treat in the same manner the hairs furnished from *Tinea tonsurans*. The fungus threads and spores are seen lying in the root sheath and also in the hair shaft.

This method of examination may be adopted for the detection of the fungus elements in Ringworm elsewhere (*Tinea barbæ*, *Tinea circinatæ*), and also in Favus and *Tinea versicolor*.

DEMONSTRATION XIX.

Anaerobic Cultures—*Bacillus Tetani*—*Bacillus Œdematis Maligni*—*Bacillus Capsulatus Aerogenes*—Inoculation of Animals with *Bacillus Tetani*, *Bacillus Œdematis Maligni* and *Bacillus Capsulatus Aerogenes*.

1. Make inoculations in the usual manner of *Bacillus tetani*, *Bacillus œdematis maligni* and *Bacillus capsulatus aerogenes* on,

(a) agar tubes (stab) to which .5% formate of soda has been added;

(b) grape sugar gelatine tubes (stab);

(c) grape sugar broth tubes.

Place the broth cultures in Novy's jar, and treat the agar and gelatine cultures by Buchner's method, as described below.

Anaerobic Culture Methods.

Anaerobiosis may be obtained in several ways. One of the most commonly employed, methods, particularly when working on a large scale, is to replace the oxygen (air) by hydrogen gas. For culture tubes and plates Novy's jars are very convenient for use with hydrogen.

2. The cultures being placed in the jar, hydrogen from a generating apparatus (Kipp's) is allowed to stream into the jar for one hour. The hydrogen is usually purified by passing through a series of wash bottles containing lead nitrate in the first, silver nitrate in second and freshly prepared pyrogallate of potash in

the third. At the end of one hour turn the stopper of the jar, which automatically seals it. The same process can be carried out by using an ordinary large tube with a rubber stopper perforated with two holes for glass tubing. After the hydrogen has been passed in for an hour, seal in the flame the glass tubing, closing first the exit tube.

Place the jar in the incubator at 37°C.

Another method of producing anaerobiosis, which is much employed is that of Buchner. It consists in the absorption of the oxygen by the use of freshly prepared pyrogallate of potash. This may be carried out in large jars, or in large test tubes.

3. Treat the agar and gelatine cultures made, by Buchner's method.

(a) In the bottom of the large test tubes furnished, place one-half inch layer of sand, and then add 1 gramme pyrogallic acid.

(b) Now place in this test tube one of the inoculated tubes and add 10cc. of 1% caustic potash solution, taking care not to soil the inoculated tube.

(c) Immediately plug firmly the large test tube with a rubber cork. Pour melted wax or paraffin about the edges of the cork, to secure perfect sealing.

The oxygen is quickly absorbed and the bacteria develop in an atmosphere of nitrogen.

Place the agar tubes in the incubator at 37°C. and keep the gelatine tubes at 20°C.

Class Demonstration.

(a) Inoculate a guinea pig subcutaneously with .25 cc. of a 72 hour broth culture of *Bacillus tetani*, tearing the tissues slightly at point of inoculation.

(b) Inoculate a guinea pig subcutaneously with .5 cc. of a broth culture of *Bacillus oedematis maligni*.

(c) Inoculate .5 cc. of a broth culture of *Bacillus capsulatus aerogenes* into the posterior auricular vein of a rabbit. Five minutes after the inoculation, kill the rabbit by a sharp blow on the back of the neck. Keep the body in a room at a temperature of 20°C and examine after 18 to 24 hours. Gas formation is seen in the veins everywhere, the viscera will be "foaming," and the animal generally emphysematous.

DEMONSTRATION XX.

Bacillus Tetani—Bacillus Œdematis Maligni—Bacillus Capsulatus Aerogenes—Examination of Animals Inoculated with Bacillus Œdematis Maligni and Bacillus Capsulatus Aerogenes.

1. Make hanging drop preparations from the broth culture of Bacillus tetani.

Slight motility can usually be detected.

2. Make cover glass films from the broth cultures of Bacillus tetani, Bacillus œdematis maligni and Bacillus capsulatus aerogenes.

After clearing in 10% acetic acid, stain in anilin gentian violet. Note the presence and position of the spores in the two former bacilli.

3. Examine carefully the guinea pig inoculated with Bacillus œdematis maligni, noting the extent and characters of the œdema. Reflect the skin in the usual manner. Make cover glass smears from the œdema fluid.

Open up the abdomen, and removing spleen, make cover glass preparations of the splenic pulp, as described under anthrax. In like manner make cover glass preparations from the heart's blood.

After drying fix these cover glass films in the formalin fluid for 1 minute.

Stain the films with eosin and methylene blue.

Examine the animal inoculated with *Bacillus capsulatus aerogenes*, and then killed and kept 18 to 24 hours.

Make cover glass preparations from the blood of the various organs. Stain with eosin and methylene blue. Capsules are seen surrounding the bacilli. These may be better demonstrated by staining in the manner described for staining the capsule of the *Pneumococcus*. (Page 28).

PART II.

BACTERIOLOGICAL ANALYSIS.

Culture Media.

For a study of the general biological characters of bacteria, growths in pure culture are required. For this growth certain nutrient materials are necessary, the nutrients varying more or less for the various species of bacteria. For the pathogenic bacteria, media approximating to the nutrients of the body are most employed. Desirable characters in culture media are clearness and ease of preparation and sterilization. The methods of preparation of the culture media in common use only, will be given.

Broth or Bouillon is much employed itself and as the basis for nutrient jellies, as agar-agar and gelatine.

One pound of lean beef-steak, freed from all fat and connective tissue bands, is chopped up in a sausage machine. 1,000 cc. of distilled water are added and the meat is lightly boiled in a covered sauce-pan for $\frac{3}{4}$ to 1 hour, occasionally being stirred. The mixture is filtered through several layers of moistened filter paper and distilled water added to make up to 1,000 cc. Place this in a flask containing 10 grammes peptone (Witte's or Fairchild's), and 5 grammes of common salt, and steam in sterilizer for $\frac{1}{2}$ hour.

Add a saturated solution of sodium carbonate, drop by drop, until the reaction becomes faintly alkaline, using litmus paper as an indicator.

(For greater accuracy in neutralization, phenolphthalein in 1% alcoholic solution may be used as an indicator. To 10 cc. of the broth, add 2 drops of the indicator and then drop in from a burette a diluted solution of sodium carbonate, till the indicator shows alkalinity. A ready calculation can then be made as to the amount of the sodium carbonate solution it will be necessary to add to the broth).

Steam again in the sterilizer for $\frac{1}{2}$ hour and then filter into a flask.

Steam this flask in the sterilizer for 20 minutes, on 3 successive days.

Instead of using beef steak, Liebig's meat extract may be employed, 3 grammes of this extract being dissolved in 1,000 cc. of water, with 10 grammes of peptone and 5 grammes of salt. The after treatment is the same as before described, except that it is better not to filter for the last time, till the broth is cold.

Glycerine Broth is ordinary broth to which 3 to 6% of pure glycerine is added, before sterilization.

Grape Sugar Broth or glucose broth is ordinary broth to which 1 to 2% of grape sugar has been added.

In Making Tubes place the broth (or liquefied jelly) in a covered funnel with stop cock or clip attachments. Add from 6 to 10 cc. to each test tube. The tubes are then re-plugged, covered with tin foil and sterilized in the steamer for 15 to 20 minutes on 3 successive days.

Gelatine. To 1,000 cc. of the stock broth add 120 grammes of gelatine ("gold label") cut up into small pieces. Dissolve carefully over a water bath, and neutralize if necessary with sodium carbonate. Place in a flask, cool down to 60°C and add the whites of two eggs. Steam for $\frac{1}{2}$ hour in the sterilizer and filter through moistened filter sheets, using a warming funnel. Steam in the sterilizer for 20 minutes on 3 successive days. Tubes are treated in same manner as broth tubes.

Grape Sugar Gelatine is stock gelatine to which 1 to 2% of grape sugar has been added.

Agar-Agar. Cut up 15 grammes of agar-agar fibre fine, and allow to soak in .5% acetic acid for 2 hours. Then wash repeatedly in water to free from all traces of the acid. Place in a flask with 1,000 cc. of stock broth, and dissolve by heating in the autoclave, up to 120°C. After removal from the autoclave, neutralize if necessary with sodium carbonate, cool to 60°C, and add the whites of two eggs dissolved in 50 cc. of water. Cook in the autoclave for 10 minutes at 110°C, and filter through moistened filter sheets, using a warming funnel. As a rule the agar runs through rapidly. If it tends to clog, immediately replace by fresh paper, keeping the unfiltered solution hot. Sterilize the filtered agar in the autoclave for 20 minutes at 120°C.

Another method which is very satisfactory is that of Ravenel, viz.,

(a) Liebig's meat extract	3 to 4 grammes.
Peptone	10 grammes.
Salt	5 grammes.
Water (distilled)	500 cc.

Boil up together for 15 minutes.

(b) Agar (prepared as above described)	15 grammes.
Distilled water	500 cc.

These are placed in a flask in the autoclave, and the temperature run up to 134°C.

Mix the two solutions, neutralize, cool to 60°C. add the whites of two eggs, and proceed as before described.

Blood Serum. Either the serum which separates from the clot or the fluids which are effused in Pleurisy or Ascites, may be employed in the making of this nutrient medium. It is one of the most difficult culture materials to handle satisfactorily. Blood is collected at the slaughter house, from cattle or sheep, in well stoppered sterile litre jars. The blood is collected after it has run for some time from the cut vessels, as it lessens the danger of contamination. The jars are then set on ice for 24 to 48 hours, and the serum which collects is syphoned off into sterile flasks, with a sterile pipette.

Several culture media are prepared from this serum.

(a) Loeffler's serum mixture. To 3 parts of the serum add 1 part of 1% grape sugar broth. Run into sterile test tubes and then place these tubes properly slanted in the serum coagulator, and expose them for 2½ to 3 hours to a carefully regulated temperature of from 90° to 95°C. This firmly sets the tubes. After-

wards they may be steamed for 15 to 20 minutes in the sterilizer on 3 successive days.

(b) Lorrain Smith's serum. To each 100 cc. of serum add 1 to 1.5 cc of a 10 % solution of caustic soda. Place in test tubes and treat as for Loeffler's serum. Or the tubes may be slanted, covered with tin foil and placed in the steamer with the lid raised and exposed to the steam for one hour. Afterwards they may be steamed for 15 to 20 minutes on 3 successive days.

(c) Kanthack and Stephen's serum agar mixture. In making this culture material it is first necessary to determine the amount of 10% caustic soda (or potash) that will be needed to transform the albumins of the serum into alkali albumins, so that no coagulation occurs on boiling. 2 cc. is about the usual amount required per 100 cc. of serum.

To 100 cc. of the serum (or serous exudate) add 2 cc. of 10 % caustic soda and 1.5 grammes of prepared agar-agar fibre. Boil up together and add 4 cc. glycerine and .5 gramme of grape sugar. Again boil and filter, using the warming funnel.

This culture material can be treated as agar. It possesses all the advantages of both serum and agar, except the clearness of the latter material.

Peptone solution of Dunham. To 100 cc. of distilled water add 1 gramme Witte's or Fairchild's peptone and .5 gramme of common salt. Boil together for 15 minutes, neutralize if necessary with sodium carbonate and again bring to a boil. Filter into flasks or

tubes, and sterilize these for 20 minutes in the steam sterilizer, on 3 successive days.

Milk. Separated milk (readily obtained from creameries) should be used, as otherwise the cream is troublesome. If the milk is fresh it may be at once sterilized, but if acid, first neutralize with sodium carbonate solution. Sterilization is best effected by steaming for 20 minutes on 3 successive days.

Litmus Milk is made by adding sufficient litmus solution to neutralized milk, to give a distinct blue tinge. The milk is then sterilized in the usual manner.

Potatoes. Select fair sized smooth potatoes and wash thoroughly. Slice off the ends, and with a cork borer, slightly smaller than the test tubes to be used, cut out cylinders from $1\frac{1}{2}$ to 2 inches long. Cut these cylinders diagonally across and allow them to soak in a .5% solution of sodium carbonate for one hour. Now place in the bottom of the test tubes to be used, a small plug of moistened absorbent cotton and then put in each, one of the potato plugs. Sterilize in the autoclave for 45 minutes at 120°C .

Preparation of Utensils.

Test Tubes. If the tubes are new, rinse several times in water and then swab out with strong nitric acid. Thoroughly rinse through water and allow to dry on a draining rack. When dry, rinse out with methylated spirit and again permit to dry.

Plug these tubes with raw cotton and sterilize in the dry air oven, at 150°C for 1 hour.

Tubes which contain old culture media or growths should, after removal of the cotton stoppers, be placed in 5% carbolic acid for 24 hours. The tubes should then be boiled in a 5% solution of washing soda for $\frac{1}{2}$ hour. This softens and allows the ready removal of any remaining material in the tubes. The tubes are then rinsed in water and treated like new tubes.

Slides. New slides need no particular treatment before use. Slides used, should be placed in turpentine for 24 hours, so as to free the cover glasses. On removal from the turpentine rinse through "waste" alcohol and then in water. Boil for a few minutes in 5% solution of washing soda, and again rinse in water, and dry. Slides may then be kept in alcohol.

Glassware. Thorough rinsing in water suffices for the cleaning of all glassware not contaminated with culture media, or like material. When so contaminated boil in 5% solution of washing soda, and then rinse in water. Flasks should be rinsed in methylated spirit and allowed to dry, before sterilization. Sterilization is effected in the hot air sterilizer at 150° to 300°C, for one hour.

Instruments. Knives, scissors, forceps and hypodermic needles are best sterilized by boiling from 5 to 10 minutes, after protecting any cutting edges by wrapping absorbent cotton about them. Sterilization may also be effected in 5% carbolic acid solution or in 1% formalin, exposing the instruments to their action for at least $\frac{1}{2}$ hour. Sterilization is readily and rapidly effected, by placing the instruments in the flame, but of

course this rapidly destroys the instrument. Boiling is certainly the most efficacious method for daily use.

Dressings. Gauze, gauze sponges, towels and aprons are best sterilized by exposing them to the action of steam in the steam sterilizer for $\frac{3}{4}$ to 1 hour. They should be first loosely wrapped in towels. If necessary, they may be dried in the hot air sterilizer at 120°C for 15 minutes.

WATER ANALYSIS.

For public health purposes a bacteriological examination of water calls for an answer to the questions, (*a*) are any pathogenic bacteria present; (*b*) has the water been polluted with sewage; (*c*) are there many bacteria present apart from sewage forms, and if so whence are they derived. Practically, the first and second questions have to be considered together, for water-borne diseases are primarily sewage-borne diseases. The third question seldom assumes much importance from a purely public health view point. Many bacteria are present in water, laden with vegetable organic matter, and this class of water would afford the basis for most examinations in answer to question (*c*).

Care should be exercised in the collection of the sample, so as to obtain a specimen which will give the general biological characters of a water. For this reason the water should be collected in cleansed sterilized jars, bottles or flasks, stoppered with a closely fitting cork. The samples should be sent at once for analysis, as even several hours in warm weather may change, to a considerable extent, the bacterial content of the water. If there is any delay before an analysis can be made, the sample should be carefully packed in ice.

Methods of Examination.

For measuring exact quantities of water a pipette graduated to .1 cc. is necessary. This should be previously sterilized, a small cotton plug being pushed in at

the upper end. Push over large end some rubber tubing with a clip. To keep this pipette sterile during the manipulations, place it in a flask of sterile water, kept boiling. Before use, it is, of course, allowed to cool.

1. (a) Add .1 cc. of the water to each of three liquefied gelatine and two agar tubes (kept at 42° C).

Pour into plates, and incubate the former at 20°C, and the latter at 37°C.

(b) Add .1 cc. of the water to each of three liquefied gelatine tubes and two agar tubes.

Pour into plates, place these in Novy's or Buchner's jars for incubation, anaerobically.

As soon as colonies appear on the agar and gelatine plates, remove and examine.

Note the number of colonies, counting the colonies by dividing the plate surface into 16 equal segments, and counting one or more segments.

Contrast the growths aerobically and anaerobically, both as to the number and character of the colonies. Nearly all sewage and pathogenic bacteria are facultative anaerobes, while the ordinary water bacteria (saphrophytes) are aerobic. Hence a large growth on the anaerobic plate is always suspicious. Good potable water, as a rule, does not contain over 100 bacteria per 1 cc., but this may run up to 1,000 and yet water be fairly good. Large numbers are always suspicious of some form of organic pollution.

Note the character of the colonies. Look for colonies suspicious of *Bacillus coli communis*, and also for liquefying forms.

Make subcultures of the varieties present on the various media, particularly any colonies which in naked eye and microscopic appearances, resemble the Colon bacillus, Typhoid bacillus or the Proteus varieties.

In sewage contamination the Colon bacillus is constantly present, usually with one or more of the putrefactive species. These, with a chemical examination indicating excess of chlorides, nitrates or albuminoid ammonia, make sewage contamination a certainty.

Tests for the Colon bacillus and putrefactive species are carried out more completely, by some of the processes later described.

2. To fermentation tubes containing sterile grape sugar broth, add 1 cc. of water. Incubate at 37°C for 24 hours. If gas is formed, *Bacillus coli communis* is probably present.

Agar and gelatine plates should be made in such a case, and the gas forming bacterium separated and identified.

3. (a) Add .1 cc of the water to each of three liquefied gelatine tubes, containing respectively .1%, .12% and .15% of carbolic acid. Pour plates and incubate at 20°C.

The majority of bacteria are checked in growth in this medium, while the Colon and Typhoid bacilli flourish well. Any colonies which appear can be further examined by subculture, to aid identification.

(b) Add .1 cc of the water to each of three liquefied tubes of Elsner's potato gelatine medium. (Elsner uses the water in which potatoes have been boiled, in place

of broth, to make gelatine. To it he adds 1% of potassium iodide). Pour plates in the usual manner. This medium has the same effect on bacterial growth as the carbolic gelatine.

4. Add .1 cc. of the water to 6 or more peptone tubes, and incubate for 24 hours at 37°C. Strips of filter paper, moistened with a solution of acetate of lead may be fixed to the stoppers. Putrefactive bacteria develop sulphuretted hydrogen during growth, and if this is formed, the lead paper is blackened. The indol test may also be applied, with and without the addition of nitrites. Plates may also be made from the surface of the fluid, and the bacteria present, separated and identified.

Peptone solution is used extensively when examining for the *Spirillum* of Asiatic Cholera. The method used is either to use the "concentrate," as described in (5), or to add to 50 cc. flasks of peptone, from 10 to 25 cc. of the water.

After 16 to 18 hours, the surface layers of the fluid are examined microscopically for vibrios. If these are present apply indol tests, and make subcultures on gelatine and agar plates, to separate and identify the species.

5. Filter through a Berkfeld or Chamberland candle, into a sterile flask, 500 cc. of the water to be examined. Place about 20 cc. of the filtered water, (which is "sterile"), into a sterile beaker, and with a sterile brush scrape the filter candle surface into the water. This "concentrates" the bacteria present, in the water. The

concentrate can then be added to carbolic gelatine, glucose broth fermentation tubes, and peptone tubes, as before described.

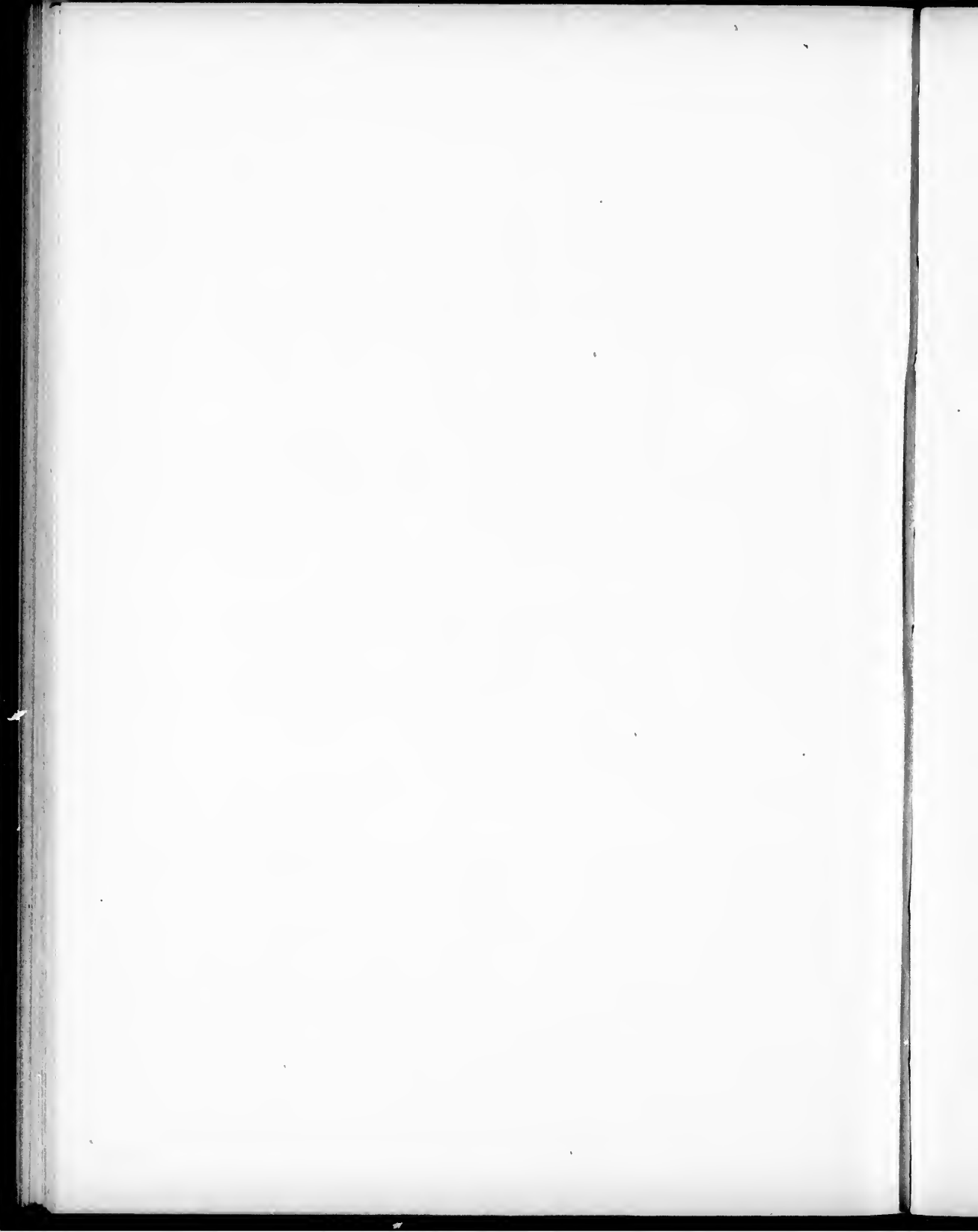
This method is particularly applicable when testing for the Typhoid and Colon bacilli, and the Cholera spirillum.

Milk Analysis.

The general principles of milk analysis are those already given under water analysis. As milk is such an excellent medium for bacterial growth, it is usually necessary to dilute the sample at least 10 times with sterile water, before making plates.

In milk analysis stress must be laid on the presence or absence of the Tubercle bacillus, it being practically the only bacterium transmitted directly from animals to man via milk. We have two tests for Tubercle bacilli in milk. (a) Microscopic examination of the sediment, after sedimentation or centrifugation, at best an unreliable method. (b) The inoculation of guinea pigs. 5 to 10 cc. of the milk is thrown into the peritoneal cavity of each of three or four guinea pigs. Three weeks after, the animals are examined for developing tubercular lesions.

From a practical standpoint, the proper application of the tuberculin test is a more ready and certain method of detecting the presence, and danger from Tuberculosis in cattle.



PART III.

CLINICAL MICROSCOPY AND DIAGNOSIS.

SECTION I.

Urine.

The sample should be taken from the entire quantity passed in 24 hours. Otherwise, select a sample passed some hours after a meal. Do not use the morning's urine for routine examination, as in this urine, least evidence of disturbance of the renal functions will be found. Putrefaction soon occurs when urine is allowed to stand, before examination. A crystal or two of thymol tends to prevent this, and does not destroy the efficacy of any of the coarser chemical or microscopical tests.

Methods of Examination.

1. *Physical examination.*

(a) Note the quantity passed in 24 hours. This is important in calculating the actual daily excretion of any normal or abnormal urinary constituents, as well as determining whether oliguria or polyuria exists.

(b) Note the color and such general characters as turbidity. Various changes in color and clearness are found both in normal and abnormal urines. These changes in normal urine depend upon the amount of

the solid constituents, putrefactive changes, and the like. The urine is variously colored by the presence of such constituents as phosphates, urates, pus, blood and bile.

(c) Take the specific gravity. By this we get (particularly in a 24 hours specimen), a good indication of the amount of solids in the urine. The usual formula given is to multiply the last two figures by 2, and so obtain the number of grammes of solids per litre.

2. *Chemical tests.*

(a) Take the reaction with litmus paper. The reaction is important in carrying out certain albumin tests. It may also indicate the nature of a sediment.

(b) Examine the sample for albumin.

If the urine be at all turbid, filter through several sheets of filter paper, before the application of the tests.

1. Use the heat and nitric acid test.

2. Apply the nitric acid test. Simon advises an excellent modification of the usual application of this method. 20 cc. of urine are placed in a conical urine glass, and 6 to 10 cc. of nitric acid are added through a pipette, passed to bottom of glass. At point of junction of the two fluids we have the formation of the usual contact ring in the presence of albumin. This ring is usually colored at bottom, rose to brick red, from the normal urinary pigment. If indican be present in excess, we have a violet ring. In biliary urine we have the usual contact play of colors (acid must contain nitrous acid as well). On standing 5 to 10 minutes, high

up in the urine a distinct white band appears, very faint normally but distinct with slight excess of uric acid. If urea be present in larger amount than 25 grammes per litre, it crystallizes out, on side of urine glass.

3. Apply the picric acid test, with Esbach's albuminometer. This is decidedly the best, day to day, clinical quantitative test.

(c) Apply the tests for sugar.

In the presence of albumin the urine should first be boiled and then filtered.

1. Use Fehling's test, the cupric sulphate and alkaline solutions being kept in separate bottles, equal parts being mixed for use. 10 cc. of this solution are reduced by .05 gm. sugar.

To apply this test quantitatively place 10 cc. of Fehling's solution, diluted with 40 cc. of water, in a porcelain capsule and bring to a boil. Add from a burette, drop by drop, the urine diluted 10 times, till all blue color is completely discharged, keeping the solution at the boiling point. Amount of diluted urine required, will contain .05 gm. of sugar.

2. If in doubt as to the accuracy of the preceding test, place urine to which a small amount of yeast (Fleischmann's) has been added, in a fermentation tube and keep at 37°C (98.6°F) for 24 hours, for gas formation.

3. Phenyl-hydrazin test. 10 cc. of the urine are boiled in a water bath for 30 minutes with 3 or 4 grammes of acetate of soda, and .3 to .5 gramme of

phenyl-hydrazin. The tube is cooled by plunging into cold water, and a microscopic examination made for phenyl-glucosazone crystals. This is a delicate test.

(d) Estimate the percentage of urea using Doremus' or larger ureometers.

These tests will be all that are usually called for in routine clinical work. Quantitative estimates may be required at times of uric acid, phosphates, chlorides, or sulphates. For these, a work on urine analysis should be consulted.

3. *Microscopic examination.*

Urine which is turbid, may be examined at once but it is better to wait till a sediment has formed. This is best effected by placing the urine in a conical glass and allowing to settle from 3 to 24 hours. The supernatant fluid is then decanted, and the sediment is placed with a pipette upon a slide and examined under the low power of the microscope. Care should be taken to withdraw the lowest sediment with the pipette. A centrifuge may be used for rapid sedimentation.

Much can be learned of the nature of a sediment by noting its color, general characters, the reaction of the urine, and whether in urine at the time of passing from bladder. The elements examined for microscopically and which appear as a rule as sediments may be divided into three classes, (1) chemical, (2) anatomical, (3) bacterial.

1. The chemical sediments consist of various crystalline and amorphous chemical bodies which may exist normally or be present abnormally in urine. We may

sub-divide them according to the reaction of urine in which found, color, and whether crystalline or amorphous.

(a) Sediments in acid urine:

Urate of soda,	amorphous,	brick red.
Uric acid,	crystalline,	bright red.
Oxalate of lime,	crystalline,	white.
Monocalcium phosphate,	crystalline (rare).	
Ammonio-magnesium phosphate,	crystalline,	white.

(found in urine becoming ammoniacal).

(b) Sediments in alkaline urine:

- Alkalinity due to fixed alkalies;
 - Basic phosphates of Ca. and Mg. amorphous, white.
 - Calcium carbonate, crystalline.
 - Dicalcium phosphate, crystalline.
- Alkalinity due to ammonia (usually fermentative):
 - Ammonium urate, crystalline, yellow.
 - Ammonio-magnesium phosphate.
 - Calcium carbonate.
 - Tricalcium phosphate.

Besides these sediments we have at times the rarer forms of chemical sediments, such as those of leucin and tyrosin, lime and magnesia soaps, cystin, xanthin and hippuric acid. It must be remembered that the presence of many of these chemical sediments, does not of necessity mean an increase in their amount in the urine. The degree of concentration, the reaction and putrefactive changes determine the presence of many of them, so that in considering their import, these points must be taken into consideration.

2. The anatomical sediments consist of cellular elements derived from the urinary passages or from the blood. They consist of various forms of epithelium, tube casts, pus cells and blood. In all normal urines a few epithelial cells, (usually derived from the bladder) are found. As a rule, too, a few leucocytes (polymorphonuclear) can be seen. Apart from these constituents, the presence of other elements means some lesion of the urinary tract.

(a) Epithelium may be derived from,

Kidney;

Renal pelvis and ureter;

Bladder and urethra;

(Vulva and vagina.)

There are no positive differential features between the epithelium from these parts, but as a rule the cells are fairly characteristic. A diagnosis cannot be based on the presence of epithelium alone, such features as albuminuria or pyuria having always to be considered.

(b) Tube casts are characteristic of lesions of the renal parenchyma. We find various forms of these casts depending on the intensity and nature of the lesion.

Tube casts may be epithelial, granular, fatty, hyaline, waxy, uratic, or consist of blood cells.

(c) Blood may be derived from any part of the urinary tract. Note must be taken of the presence of clots, the nature of the admixture with urine and the presence of ureteral or true blood casts, in determining the source of the haemorrhage.

(d) Pus cells (leucocytes) are found in all irritative or inflammatory lesions of the urinary tract. Only rarely are they found alone, (rupture of abscess into passages). As a rule we find, and must consider the presence of albumin, epithelium or casts, as well as the chemical characters of the urine, in determining the site of lesion.

3. Bacteria in the urine may be derived from the urinary passages or may enter the urine after its passage from the body (putrefaction).

The bacteria which may be found in affections of the urinary tract are as follows:

(a) The Tubercle bacillus is found in Tuberculosis of any part of the urinary tract. It is examined for as described at page 32.

(b) Occasionally certain bacteria which may be excreted through the kidney, multiply in the passages exciting a bacteriuria. This is seen occasionally after Typhoid fever and in some infections with the *Bacillus coli communis* and more rarely with other microbes during the course of a septicaemia or pyaemia.

(c) Bacteria may make their way to the bladder and upper urinary passages by being introduced through urethra on catheters, etc. This usually excites the condition of ammoniacal cystitis and by extension gives rise to a form of "surgical" kidney.

These three forms must be distinguished from the bacteria which enter and cause putrefaction of the urine after its passage from the bladder.

SECTION II.

Blood.

Blood examinations require the use of special instruments and reagents. The instruments which will be needed besides a microscope, are a haemoglobinometer (Fleischl's), a haemacytometer (Thoma's) and a spear headed needle for obtaining blood. A haematocrit and centrifuge are very useful when much blood work has to be undertaken.

The following reagents will be required:

Toison's dilution fluid,

Glycerine	30 parts
Sodium sulphate	8 "
Sodium chloride	1 "
Methyl violet025 "
Water	160 "

Benzol and chloroform, and a mixture of these two having a specific gravity of 1055.

Ehrlich's triple stain,

Saturated aqueous solution acid fuchsin	2½ parts
Distilled water	2 "
Saturated aqueous solution orange G.	6 "
" " " methylene green	6½ "

Add the last slowly, drop by drop, constantly stirring.

Absolute alcohol	5 parts
Distilled water	10 "

If the methylene green precipitates add more acid fuchsin.

Eosin .5% solution in 70% alcohol.

Loeffler's methylene blue.

Ehrlich's eosin-haematoxylin stain,

Haematoxylin	4-5 parts
Alcohol, glycerine, and water	100 " each
Glacial acetic acid	20 "
Alum	to saturation.

Allow to ripen in sun 5 or 6 weeks, and add 1% of eosin.

In blood examination the points to be ascertained are the specific gravity, the amount of haemoglobin and the number and characteristics of the red and white blood cells. Examination bacteriologically by cover glass preparations and cultures may also be required.

The blood may be obtained from the lobe of the ear, root of the finger nail or the ball of the finger, the first being undoubtedly the best situation. The part should be washed with soap and water, then with a mixture of alcohol and ether before puncturing. The first drop of blood which exudes should be wiped away with sterile gauze. No pressure should be made to obtain the blood.

Specific Gravity. Allow a drop of blood to fall into a cylindrical vessel containing the benzol-chloroform mixture. If it floats add more benzol, if it sinks add chloroform, till the drop remains stationery about the middle of the fluid. Take the specific gravity of the mixture and this will be the reading for the blood.

Estimation of Haemoglobin. After arranging the apparatus (Fleischl's) cover the bottom of one of the cells with distilled water. Fill the capillary pipette with blood, taking care not to soil its exterior, and to have the tube just full. Rinse out the pipette in the water of the cell, and by dropping on more distilled water. Fill the cell almost full and with the handle of the pipette thoroughly mix the contents of the cell. Both cells are now carefully filled with water till both present a convex surface. Using an oil or gas lamp, (not daylight) compare the tint of the blood cell with that of the standard bar under the water cell, moving the bar till the two tints are the same. Read off the percentage of normal on the bar index.

Estimation of the Red Corpuscles. Carefully fill the pipette of the haemocytometer up to 1 with blood. At once draw in dilution fluid till mark on bulb, 101, is reached. Mix the blood and the fluid by twirling the pipette between the fingers for a minute. After blowing out the fluid that fills the tube of the pipette, place a drop of the mixture in the bulb, on the centre of the counting slide. Place over this a cover glass, taking care not to allow the blood to overflow the cell edges. Examine under the microscope and finding the ruled squares, count the red corpuscles on at least 40 squares. This should be repeated several times and an average taken. As each ruled square is $\frac{1}{4000}$ c. mm. we can readily calculate the number of corpuscles per c. mm., by the following formula:

Number of corpuscles per 1 c. mm. =

$$\frac{\text{No. of corpuscles} \times 4,000 \times 100 \text{ (dilution)}}{\text{No. of squares.}}$$

Normally the red blood cells number between 5,000,000 and 5,500,000 per c. mm.

By this method of examination, information can also be obtained of the size and shape of the red corpuscles.

Estimation of White Corpuscles. The white corpuscles may be estimated at the same time as the red, by counting at least 200 squares.

It is better to estimate them separately, using the large pipette of the haemocytometer. Blood is drawn into this pipette up to 1 and then the bulb is filled up to 11 with a .4% solution of acetic acid. The acid destroys the red corpuscles leaving the white cells. Examination is then made in the same manner as for the red corpuscles. Normally there are on an average 10,000 white corpuscles per c. mm.

The Haematocrit may also be used to determine the numbers of red and white cells. The blood is drawn into the graduated pipette and at once centrifuged, at 10,000 revolutions per minute, for 3 minutes. Normally the red corpuscles will stand at 50 (on a scale of 100) so that 1% = 100,000 corpuscles. To calculate the number of red corpuscles one simply adds five ciphers to the percentage reading. The leucocytes are found on the surface of the red discs, as a narrow white band. Any increase in their numbers will be detected by an increase of this band.

Microscopic Examination of Blood Films.

(a) Direct examination of moist films is called for, in determining poikilocytosis, leucocytosis, and in examining for the malarial plasmodium.

Moist preparations are made by touching the blood drop with a thoroughly clean cover glass, and then inverting this upon a slide. Only a small drop must be taken on the cover glass so that it will spread out in a single layer on the slide. To prevent drying, the cover glass may be ringed with vaseline.

(b) Examination of films dried and stained, is of much wider application, being employed to determine the same characters as the moist films, and in addition giving the forms and relative proportions of the white cells and the staining characters of the red corpuscles.

The blood films are prepared by touching one cover glass to the blood, so as to obtain a small drop, then dropping on this cover glass a second, and sliding the two apart. The films are dried in the air and are then ready for fixation. This may be effected by heat or in certain fixing fluids. Heat fixation gives the best results. The cover glasses may be placed in an oven for 2 hours at a temperature of 110°C . As this method cannot always be carried out, a cheap and effective substitute, is made by the use of a long triangle of brass, about .5 cm. in thickness. A gas or spirit lamp flame is applied to the apex and after 5 or 10 minutes tests are made for the boiling point on the triangle. The cover glasses are placed 2 to 3 cm. in front of this

point, where the temperature averages between 110° and 120°C , and are allowed to remain for 2 hours.

A more rapid method of fixation is to place the films for 1 minute in a solution made by taking 1 part of formalin, 9 parts water and 90 parts of alcohol. This method produces almost as satisfactory results as are obtained by heat fixation.

Staining of the Films. (a) For general purposes Ehrlich's triple stain is perhaps the best. It stains the red corpuscles orange, the eosinophile granules red, the neutrophile granules violet, nuclei of red corpuscles (when present) greenish black, nuclei of leucocytes greenish blue, the nuclei of the lymphocytes being, as a rule, stained less deeply than the others. In using this stain care must be taken not to shake it up. The stain is best withdrawn on a glass rod and the films covered for 6 to 8 minutes, then carefully washed in water, dried and mounted in Canada balsam.

(b) Eosin and Loeffler's methylene blue.

Place the films in eosin 2 minutes, then wash and dry. Place in methylene blue for $\frac{1}{2}$ to 1 minute, wash dry and mount in Canada balsam.

The red corpuscles and eosinophile granules are stained red, nuclei blue, malarial parasites blue.

(c) Ehrlich's eosin-haematoxylin stain.

The films are placed in this stain for 24 hours, exposed to light, and are then washed, dried and mounted.

The red corpuscles and eosinophile granules are stained red, nuclei of red discs and lymphocytes, black, nuclei of other white cells, bluish black.

SECTION III.

Gastric Contents.

Examinations of the gastric contents are required to obtain information of the condition and digestive power of the normal gastric juices, and to determine the presence of any abnormal constituents, such, for instance, as the products of fermentation. Accurate information, upon which conclusions can be based, is only to be obtained after several examinations and the use of test meals on a food free stomach, the contents being withdrawn at a set period.

The most commonly employed test meal is the test breakfast of Ewald and consists of 35 to 40 grammes white bread, and 300 cc. of water, or tea without milk or sugar. This is withdrawn one hour afterward, if possible, without the use of water. If water has to be used, measured small quantities only should be employed. At the expiration of one hour, normally about 40 cc. of contents can be withdrawn, and in this we can demonstrate the presence of free and combined hydrochloric acid, pepsin, rennet, peptone and glucose.

Boas advises a special test meal in cases of suspected carcinoma of stomach. It consists of from 350 to 400 cc. of oatmeal gruel made with water and salt, and given at night, after lavage of the mouth and stomach. Eight or ten hours afterwards the contents are expressed. The meal is given to prevent the introduction of lactic acid in the food, and the tests are made for lactic acid alone.

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The examinations made of the gastric contents are physical, chemical and microscopical.

1. *Physical examination.*

(a) Measure the *amount* of contents obtained. More than 40 cc. indicates loss of motor and absorptive power of the stomach wall

(b) Note the *color*, which is normally light yellow or brownish yellow. The presence of blood, bile or much mucus, changes the color.

(c) Note the *odor*. If fatty acids as acetic and butyric acids are present, they can readily be detected in this manner.

(d) Note the amount of *mucus*. This is increased in nearly all forms of gastric affections, but more particularly so in "chronic" gastritis. The presence of food fragments, portions of mucous membrane and the like, must also be noted.

2. *Chemical examination.*

In a chemical examination of the gastric contents the following reagents will be required.

Congo red solution. A dilute aqueous solution of Congo red powder of a brick red color.

Phloroglucin-vanillin solution of Gunzburg. Phloroglucin 2 grammes, vanillin 1 gramme, absolute alcohol 30 cc.

Dimethyl-amido-azo-benzol. .5% alcoholic solution.

Phenolphthalein. 1% alcoholic solution.

Alizarin. 1% aqueous solution.

Uffelmann's reagent. 3 drops of saturated aqueous solution of the perchloride of iron are mixed with 3 drops of pure carbolic acid, and water added till an amethyst blue solution is obtained.

Decinormal sodium hydrate solution. Contains 4 grammes of Na OH in a litre of water. 1 cc. of the decinormal solution neutralizes .00,365 grammes of HCl.

The chemical tests are applied to the filtrate, one-half of the contents being filtered.

(a) Determine the *acidity* with litmus paper.

Acidity may be due to HCl free or combined, to acid salts, or to the presence of such fermentative acids as lactic, acetic or butyric.

(b) If acid, determine the *total acidity* by placing 5 cc. of the filtered contents in a capsule, adding one or two drops of phenolphthalein solution, and then carefully adding from a burette, decinormal sodic hydrate till completely neutralized. This will be indicated when no further intensification of the red color occurs, on the addition of more sodic hydrate. Normally from 40 to 65 cc. of the decinormal sodic hydrate solution are required to neutralize 100 cc. of the gastric contents.

(c) If acid, is the *acidity due to free acids* or acid salts. To determine this add a drop of the gastric contents to a drop of the Congo red solution. If free acids are present an azure blue coloration appears.

(d) If free acid is present, determine if *free HCl* exists.

(1) To a drop of the filtrate in a porcelain capsule add a drop of Gunzburg's phloroglucin-vanillin solu-

tion. Carefully evaporate high over the flame. If free HCl is present a rose red ring develops at margin of evaporating drop. This test will determine the presence of .05 parts of free HCl per mille.

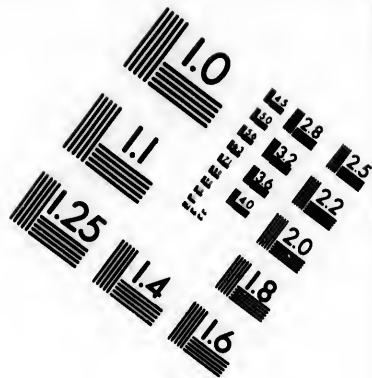
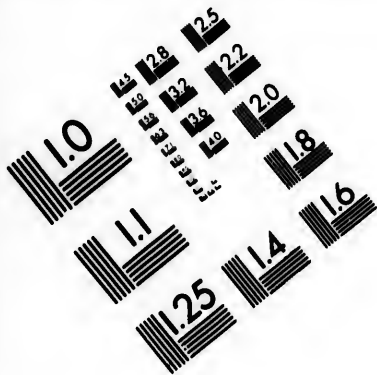
(2) To a drop of the filtrate on a porcelain capsule, add a drop of the dimethyl-amido-azo-benzol solution. This gives a red coloration, in the presence of free HCl when present in .02 parts or over, per mille. It is a more delicate test than Gunzburg's and can be used to determine the amount of free HCl present.

(e) While in practice it is found that in the presence of free HCl, tests for *lactic acid* may be disregarded, yet when free acid is present a test should be made for lactic acid.

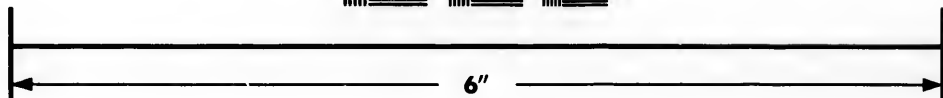
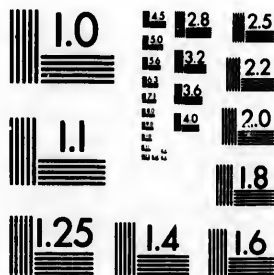
Place in a graduate or test tube about 20 cc. of Uffelmann's reagent. Add drop by drop from a pipette the filtered stomach contents, a lemon yellow precipitate falls in the presence of lactic acid.

This test, while all that need be applied for clinical purposes, is not always accurate as it is interfered with by excess of HCl, presence of acetic and butyric acids, alcohol or much glucose. A more satisfactory method is to shake up 10 cc. of the filtrate with 30 cc. of ether, repeating twice, and removing the ether with a pipette. The ether is then carefully evaporated, the residue extracted with water and used to make the test.

(f) The *fatty acids* particularly acetic and butyric acids are at times present as fermentative products. The amount of these acids usually rises and falls with the amount of lactic acid. Their odor is the best qualitative test.



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10

(g) When the relative proportions of the various constituents, which produce acidity, are to be determined the following method gives quite accurate results. 10 cc. of the filtered contents are placed in a beaker and decinormal sodic hydrate solution is added, drop by drop, till a drop taken out with a small platinum loop, no longer gives a reaction with the dimethyl-amido-azobenzol solution. The reading on the burette gives the amount of solution required to neutralize the free HCl (which has the strongest affinity for the alkaline solution).

Now add a loop of the mixture to a drop of Congo red solution. If this still indicates acidity, add more of the soda solution till the Congo red no longer indicates. The reading on the burette gives the amount of alkaline solution required to neutralize the free organic acids present.

A loop of the contents is next added to a drop of alizarin (indicates alkalinity), and if there is no violet reaction, add the soda solution till this develops. The reading on the burette gives the amount of the soda required to neutralize the acid salts.

If contents are still acid to phenolphthalein, add more of the soda solution, till the rose-red color completely develops. The reading on the burette gives the amount of solution required to neutralize the combined HCl.

If there is no free HCl, nor free acid, titration can be commenced at the corresponding portion of the process just described.

(h) Test for *pepsin* by determining the digestive powers of the juice. In the absence of free HCl no pepsin will be found and tests must be directed towards its zymogen, pepsinogen.

Add a small, shaving 1 cm. square and 1 mm. thick, of coagulated egg albumin to 10 cc. of the filtrate, and keep at 37°C, one to two hours. If there is no free HCl present, add 3 or 4 drops of a 25% solution of HCl, so as to transform any pepsinogen into pepsin. After two hours the shaving ought to be digested.

Quantitative estimation of the pepsin is carried out by making a series of dilutions and testing the digestive powers of these dilutions. This procedure is rarely called for in clinical practice.

(i) Tests for the *milk curdling ferment*, "rennet" and its zymogen.

1. In testing for rennet add 3 to 5 drops of the filtrate to 10 cc. milk and keep at 37°C. If rennet is present the milk clots in 10 to 15 minutes.

(2) If no coagulation occurs, test for the rennet zymogen, by neutralizing some of the gastric contents with lime water, and then adding 3 to 5 drops, to the milk, when if the zymogen is present, coagulation occurs in 10 to 15 minutes. Quantitative tests can be carried out by a series of dilutions as is done for pepsin, but are rarely required clinically.

(j) Tests for the condition of the various food constituents of the test meal, may also be carried out. Practically, tests are required only for presence of pepsin, propeptones, and the stage of starch digestion.

1. Test for *propeptones* (or albumoses), by adding to 5 cc. of the filtrate, a saturated solution of sodium chloride, when any propeptone is precipitated. The more turbid the fluid becomes, the greater the quantity of propeptone. If no precipitate falls, a drop of acetic acid (10% solution) may be added when, if present, propeptone falls. This addition will only be required in the absence of free acid.

2. Test for *peptones* by adding to the filtrate, after propeptones have been precipitated and filtered out, an equal quantity of caustic soda solution and a few drops of 1% solution of copper sulphate. A violet red or purplish color (biuret) is produced, if peptone is present.

(9) *Starch* should be so far changed in the gastric contents as not to give either the reaction for starch or for erythro-dextrin. Test for these, by adding to several drops of the filtrate, a drop of tincture of iodine. Starch, if present, will give a blue reaction, erythro-dextrin, a purplish red.

3. *Microscopical examination.*

Portions of the unfiltered contents are examined under the microscope, with the low and high lenses and with the oil immersion lens. By this means we determine the nature of any food fragments, (starch granules, milk and fat globules), the presence of blood, pus, epithelium, and bacteria, including sarcinae and yeasts.

Any fragments resembling portions of a new growth may be sectioned and examined in the usual manner.

Cultures may be made on the various laboratory media to separate out and identify any bacteria present.

