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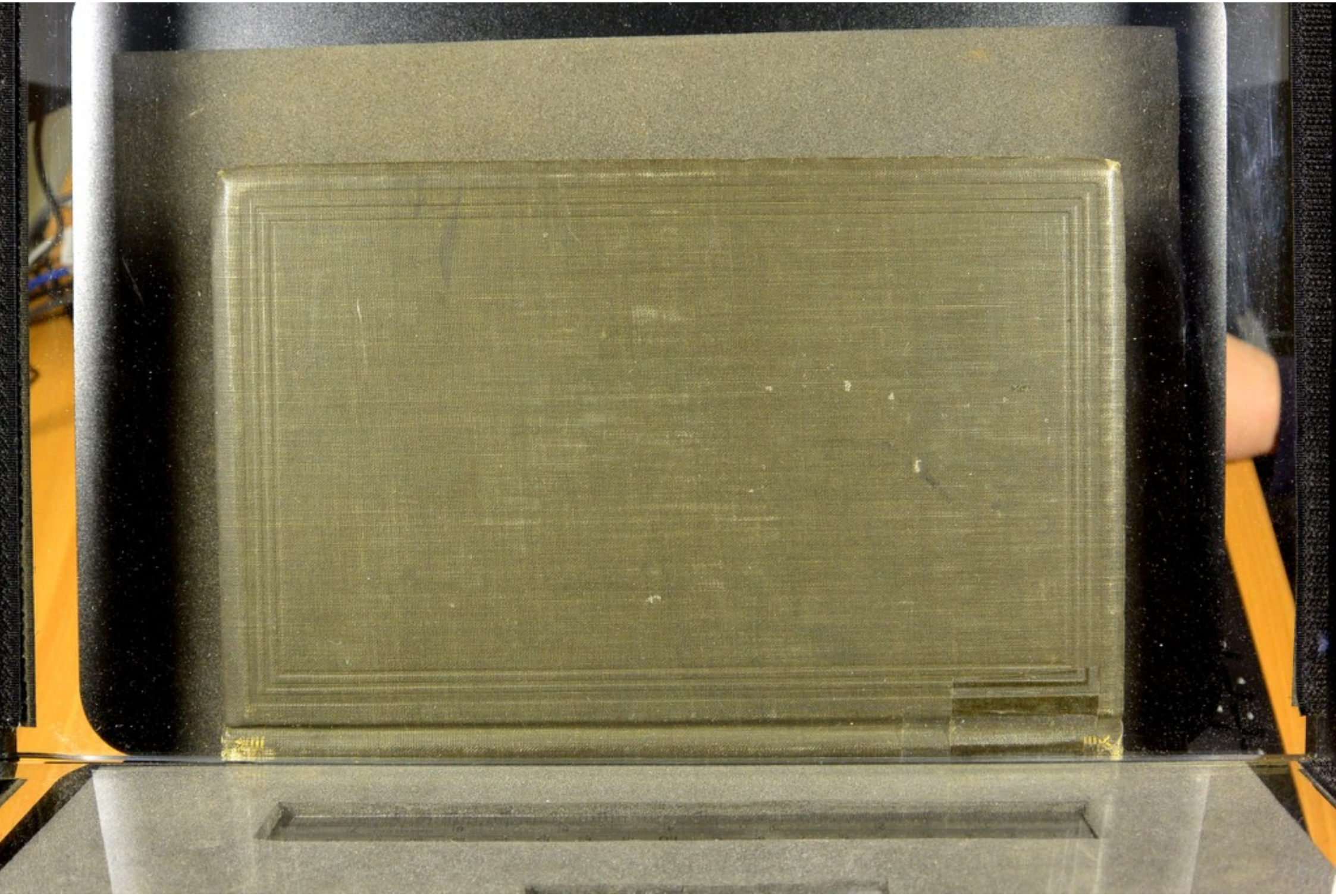

THE ELEMENTS OF BACTERIOLOGICAL TECHNIQUE

A LABORATORY GUIDE

FOR THE

MEDICAL, DENTAL, AND TECHNICAL STUDENT

BY

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WITH 170 ILLUSTRATIONS

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TO THE MEMORY OF

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Physician to Guy's Hospital and Lecturer on Bacteriology in the Medical School, and Physician to the London Fever Hospital

MY TEACHER, FRIEND, AND CO-WORKER

PREFACE.

In the following pages I have endeavoured to arrange briefly and concisely the various methods at present in use for the study of bacteria, and the elucidation of such points in their life-histories as are debatable or still undetermined.

Of these methods, some are new, others are not; but all are reliable, only such having been included as are capable of giving satisfactory results even in the hands of beginners. In fact, the bulk of the matter is simply an elaboration of the typewritten notes distributed to some of my laboratory classes in practical and applied bacteriology; consequently an attempt has been made to present the elements of bacteriological technique in their logical sequence.

I make no apology for the space devoted to illustrations, nearly all of which have been prepared especially for this volume; for a picture, if good, possesses a higher educational value and conveys a more accurate impression than a page of print; and even sketches of apparatus serve a distinct purpose in suggesting to the student those alterations and modifications which may be rendered necessary or advisable by the character of his laboratory equipment.

The excellent and appropriate terminology introduced by Chester in his recent work on "Determinative Bacteriology" I have adopted in its entirety, for I consider that it only needs to be used to convince one of its extreme utility, whilst its inclusion in an

elementary manual is calculated to induce in the student habits of accurate observation and concise description.

With the exception of Section XVII,—"Outlines for the Study of Pathogenic Bacteria,"—introduced with the idea of completing the volume from the point of view of the medical and dental student, the work has been arranged to allow of its use as a laboratory guide by the technical student generally, whether of brewing, dairying, or agriculture.

So alive am I to its many imperfections that it appears almost superfluous to state that the book is in no sense intended as a rival to the many and excellent manuals of bacteriology at present in use, but aims only at supplementing the usually scanty details of technique, and at instructing the student how to fit up and adapt apparatus for his daily work, and how to carry out thoroughly and systematically the various bacterioscopical analyses that are daily demanded of the bacteriologist by the hygienist.

Finally, it is with much pleasure that I acknowledge the valuable assistance received from my late assistant, Mr. J. B. Gall, A.I.C., in the preparation of the section dealing with the chemical products of bacterial life, and which has been based upon the work of Lehmann,

JOHN W. H. EYRE.

GUY'S HOSPITAL, S. E.,
September, 1902.

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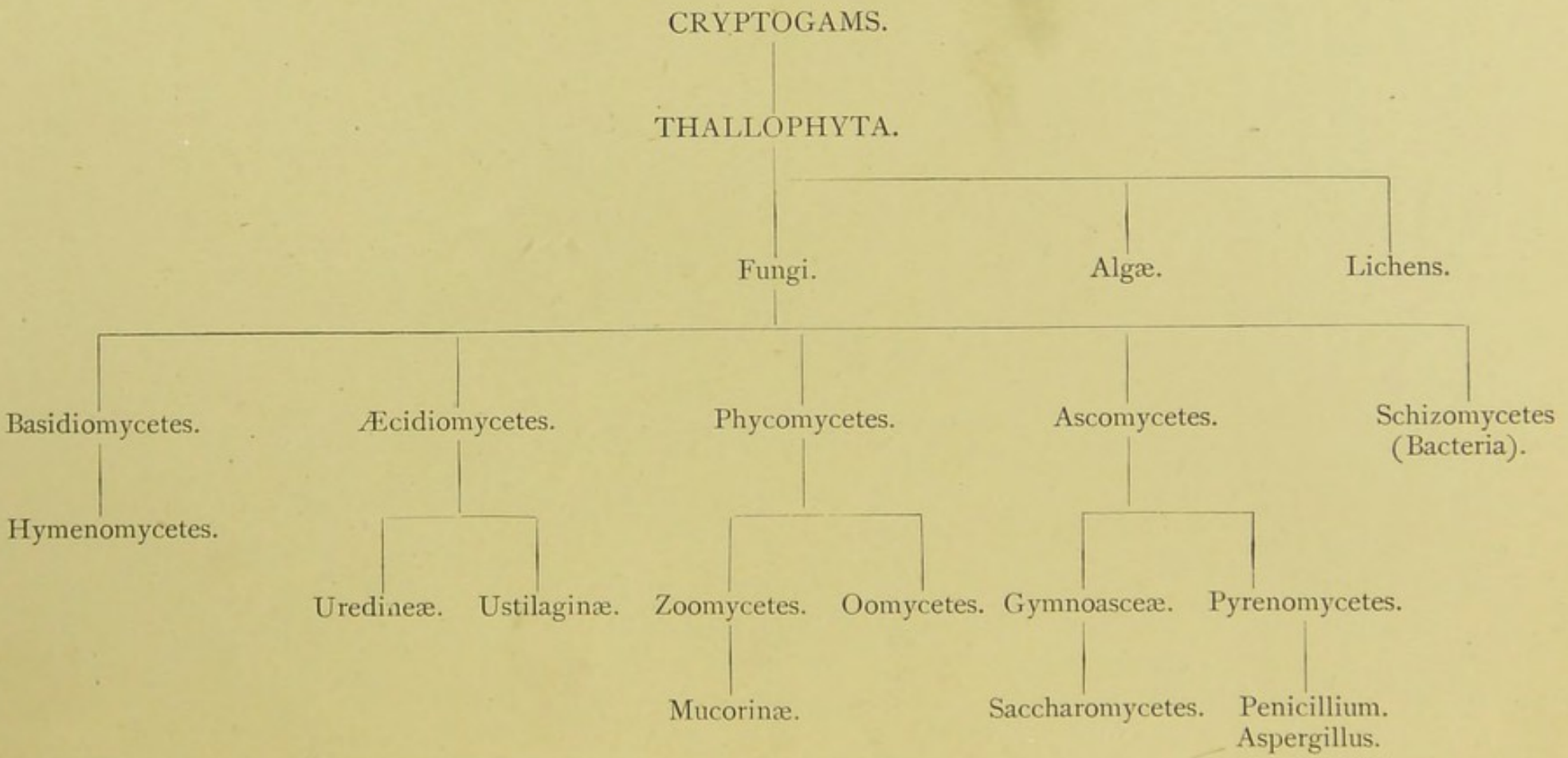
THE POSITION OF BACTERIA IN THE VEGETABLE KINGDOM.



BACTERIOLOGICAL TECHNIQUE.

I. GLASS APPARATUS IN COMMON USE.

THE equipment of the bacteriological laboratory, so far as the glass apparatus is concerned, differs but little from that of a chemical laboratory, and the cleanliness of the apparatus is equally important. The glassware comprised in the following list, in addition to being clean, must be stored in a sterile or germ-free condition.

Test-tubes.—It is convenient to keep several sizes of test-tubes in stock, to meet special requirements, viz.:

1. 18 by 1.5 cm., to contain media for ordinary tube cultivations.
2. 18 by 1.3 cm., to contain media used for pouring plate cultivations, and also for holding sterile "swabs."
3. 18 by 2 cm., to contain wedges of potato, beet-root, or other vegetable media.
4. 13 by 1.5 cm., to contain inspissated blood-serum.

The tubes should be made from the best German potash glass, "blue-lined," stout and heavy, with the edge of the mouth of the tube *slightly* turned over, but not to such an extent as to form a definite rim. (Cost about $4.25, or 9 shillings per gross.) Such tubes are sufficiently stout to resist rough handling, do not usually break if accidentally allowed to drop, can be cleaned, sterilised, and used over and over again,

and by their length of life fully justify their initial expense.

A point to be noted is that the manufacturers rarely turn out such tubes as these absolutely uniform in calibre, and a batch of 18 by 1.5 cm. tubes usually contains such extreme sizes as 18 by 2 cm. and 18 by 1.3 cm. Consequently, if a set of standard tubes is kept for comparison, each new supply of 18 by 1.5 cm. tubes may be easily sorted out into these three sizes, and so simplify ordering.

5. 5 by 0.9 cm., for use in the inverted position inside the tubes containing carbohydrate media, as gas-collecting tubes.

6. 5 by 0.5 cm., for sedimentation reactions, etc.

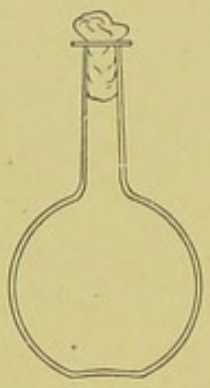


Fig. 1.—Bohemian flask.

Fig. 2.—Pear-shaped flask.

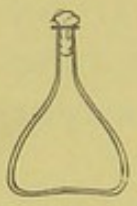


Fig. 3.—Erlenmeyer flask (narrow neck).

These tubes may be of common thin glass, "unrimmed," as less than two per cent. are fit for use a second time.

Bohemian Flasks (Fig. 1).—These are the ordinary flasks of the chemical laboratory. A good variety, ranging in capacity from 250 to 3000 c.c., should be kept on hand. A modified form, known as the "pear-shaped" (Fig. 2), is preferable for the smaller sizes—*i. e.*, 250 and 500 c.c.

Erlenmeyer's Flasks (Fig. 3).—Erlenmeyer's flasks of 75, 100, and 250 c.c. capacity are extremely useful. For use as culture flasks care should be taken to select

only such as have a narrow neck of about 2 cm. in length.

Kolle's Culture Flasks (Fig. 4).—These thin, flat flasks (to contain agar or gelatine, which is allowed to solidify in a layer on one side) are extremely useful on account of the large nutrient surface available for growth. A surface cultivation in one of these will yield as much growth as ten or twelve "oblique" tube cultures. The wide mouth, however, is a disadvantage, and for many purposes thin, flat culture bottles (Fig. 5) are to be preferred.

Filter Flasks or Kitasato's Serum Flasks (Fig. 6).—Various sizes, from 250 to 2000 c.c. capacity. These

Fig. 4.—Kolle flask.

Fig. 5.—Culture bottles.

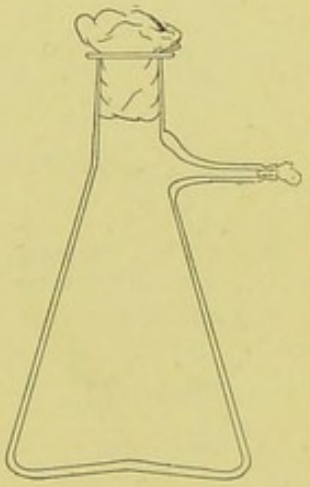


Fig. 6.—Filter flask.

must be of stout glass, to resist the pressure to which they are subjected, and must be thoroughly well annealed, in order to withstand the temperature necessary for sterilisation.

Petri's Dishes or "Plates" (Fig. 7, *a*).—These have now completely replaced the rectangular sheets of glass introduced by Koch for the plate method of cultivation. Each "plate" consists of a pair of circular discs of glass with sharply upturned edges, thus forming shallow dishes, one of slightly greater diameter than the other, and so, when inverted, forming a cover or

cap for the smaller. Plates having an outside diameter of 10 cm. and a height of 1.5 cm. are the most generally useful. Such plates are sterilised and stored in batches of eighteen in cylindrical copper boxes with a "pull-off" lid, 30 cm. high by 12 cm. diameter. Inside the box is a copper stirrup with a circular bottom, upon which the plates rest, and by means of which each can be raised in turn to the mouth of the box (Fig. 8).

Capsules (Fig. 7, *b* and *c*).—These are Petri's dishes of smaller diameter but greater depth than those termed

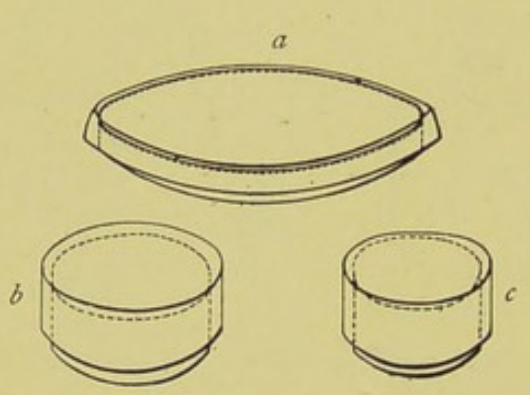


Fig. 7.—Petri dish and capsules.

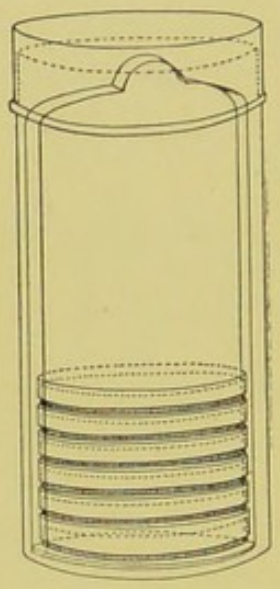


Fig. 8.—Plate box with stirrup.

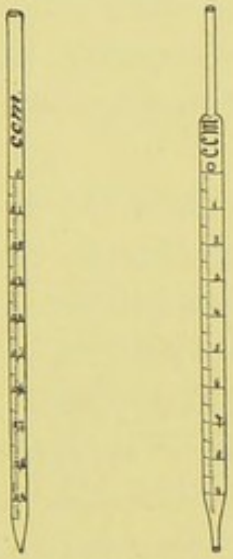
plates. Two sizes will be found useful—viz., 4 cm. diameter by 2 cm. high, capacity about 14 c.c.; and 5 cm. diameter by 2 cm. high, capacity about 25 c.c. These are stored in copper cylinders of similar construction to those used for plates, but measuring 20 by 6 cm. and 20 by 7 cm., respectively.

Graduated Pipettes.—Several varieties of these are required, viz.:

1. Pipettes of 1 c.c. capacity graduated in 0.1 c.c.
2. Pipettes of 1 c.c. capacity graduated in 0.01 c.c. (Fig. 9, *a*).

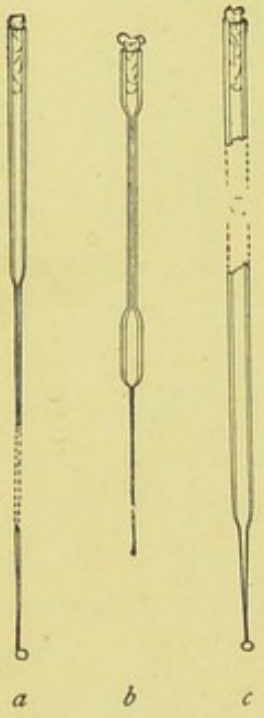
3. Pipettes of 10 c.c. capacity graduated in 0.1 c.c. (Fig. 9, *b*).

These should be about 30 cm. in length (1 and 2 of fairly narrow bore), graduated to the extreme point, and having at least a 10 cm. length of clear space between the first graduation and the upper end. Each variety should be stored in a separate cylindrical copper case some 36 by 6 cm., with "pull-off" lid, upon which is stamped, in plain figures, the capacity of the contained pipettes.



a *b*

Fig. 9.—Measuring pipettes, *a* and *b*.



a *b* *c*

Fig. 10.—Pasteur's pipettes, *a*, *b*, *c*.

Capillary Pipettes or Pasteur's Pipettes (Fig. 10, *a*).—These little instruments are invaluable, and a goodly supply should be kept on hand. They are prepared from soft glass tubing of various-sized calibre in the following manner: Hold a 14 cm. length of glass tube by each end, and whilst rotating it heat the central portion in the Bunsen flame until the glass is red hot and soft. Now remove it from the flame and steadily pull the ends apart, so drawing the heated portion out into a capillary tube; break the capillary portion

at its centre, seal the broken ends in the flame, and round off the edges of the open end of each pipette. A loose plug of cotton-wool in the open mouth completes the capillary pipette. After a number have been prepared, they are sterilised and stored in batches, either in metal cases similar to those used for the graduated pipettes or in large-sized test-tubes—sealed ends downwards and plugged ends towards the mouth of the case.

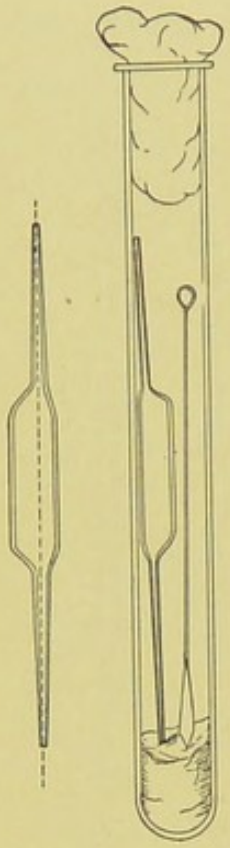


Fig. 11.—Blood pipettes and hare-lip pin in a test-tube.

A modification of this pipette, in which a constriction or short length of capillary tube is introduced just below the plugged mouth (Fig. 10, *b*), will also be found extremely useful in the collection and storage of morbid exudations.

A third form, where the capillary portion is about 4 or 5 cm. long and only forms a small fraction of the entire length of the pipette (Fig. 10, *c*), will also be found useful.

"Blood" Pipettes (Fig. 11).—Special pipettes for the collection of fairly large quantities of blood (as suggested by Pakes) should also be prepared. These are made from *soft* glass tubing of 1 cm. bore in a similar manner to the Pasteur pipettes, except that a blowpipe flame must be used in order to obtain the sharp shoulder at either end of the central bulb. The terminal tubes must retain a diameter of at least 1 mm., in order to avoid capillary action during the collection of the fluid.

For sterilisation and storage each pipette is placed inside a test-tube, resting on a wad of cotton-wool, and the tube plugged in the ordinary manner. As these tubes are used almost exclusively for blood work,

it is usual to place a lance-headed hare-lip pin inside the tube so that the entire outfit may be sterilised at one time.

Graduated Capillary Pipettes (Fig. 12).—These should also be made in the laboratory,—from manometer tubing,—of simple, convenient shape, and graduated by the aid of a 1 c.c. pipette (in hundredths) to contain such quantities as 10, 50, and 90 c.mm., and carefully marked with a writing diamond. These, previously sterilised in large test-tubes, will be found extremely useful in preparing accurate percentage solutions, when only minute quantities of fluid are available.

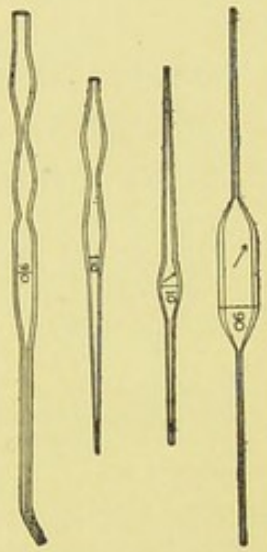


Fig. 12.—Capillary graduated pipettes.

Sedimentation Tubes (Fig. 13).—These are prepared from 10 cm. lengths of narrow glass tubing by sealing one extremity, blowing a small bulb at the centre, and plugging the open end with cotton-wool; after sterilisation the open end is provided with a short piece of rubber tubing and a glass mouthpiece. When it is necessary to observe sedimentation reactions in very small quantities of fluid,

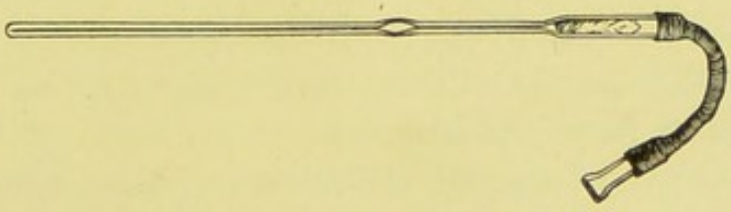
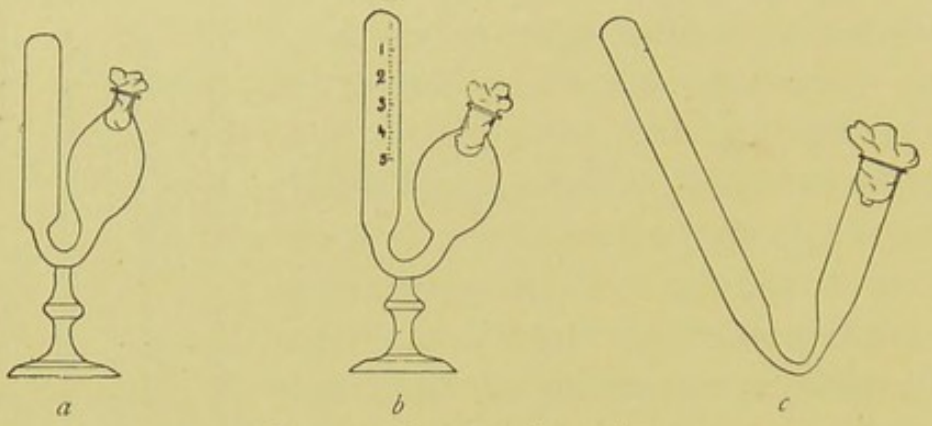


Fig. 13.—Sedimentation tube.

these tubes will be found much more convenient than the 5 by 0.5 cm. test-tubes previously mentioned.

Fermentation Tubes (Fig. 14).—These are used for the collection and analysis of the gases liberated from the media during the growth of some varieties of bac-

teria and may be either plain (*a*) or graduated (*b*). A simple form (Fig. 14, *c*) may be made from 14 cm. lengths of soft glass tubing of 1.5 cm. diameter. The Bunsen flame is applied to a spot some 5 cm. from one end of such a piece of tubing and the tube slightly drawn out to form a constriction, the constricted part



a *b* *c*

Fig. 14.—Fermentation tubes.

is bent in the bat's-wing flame, to an acute angle, and the open extremity of the long arm sealed off in the blowpipe flame. The open end of the short arm is rounded off and then plugged with cotton-wool, and the tube is ready for sterilisation.

CLEANING OF GLASS APPARATUS.

All glassware used in the bacteriological laboratory must be thoroughly cleaned before use, and this rule applies as forcibly to new as to old apparatus, although the methods employed may vary slightly.

To Clean New Test-tubes.—

1. Place the tubes in a bucket or other convenient receptacle, fill with water and add a handful of "sapon" or other soap powder. See that the tubes are full and submerged.

2. Fix the bucket over a large Bunsen flame and boil for thirty minutes.

3. Cleanse the interior of the tubes with the aid of test-tube brushes, and rinse thoroughly in cold water.

4. Invert the tubes and allow them to drain completely.

5. Dry the tubes and polish the glass inside and out with a soft cloth, such as selvyt.

New flasks, plates, and capsules must be cleaned in a similar manner.

To Clean New Graduated Pipettes.—

1. Place the pipettes in a convenient receptacle, filled with water to which soap powder has been added.

2. Boil the water vigorously for twenty minutes over a Bunsen flame.

3. Rinse the pipettes in running water and drain.

4. Run distilled water through the pipettes and drain.

5. Run rectified spirits through the pipette and drain as completely as possible.

6. Place the pipettes in the hot-air oven (*vide* page 35), close the door, open the ventilating slide, and run the temperature slowly up to about 80° C. Turn off the gas and allow the oven to cool.

Or 6*a*. Attach each pipette in turn to the rubber tube of the foot bellows, or blowpipe air-blast, and blow air through the pipette until the interior is dry.

Glassware that has already been used is regarded as *infected*, and is treated in a slightly different manner.

Infected Test-tubes.—

1. Pack the tubes in the wire basket of the autoclave (having previously removed the cotton-wool plugs, caps, etc.), in the vertical position, and before replacing the basket see that there is a sufficiency of water in the bottom of the boiler. Now attach a piece of rubber tubing to the nearest water tap, and by means of this fill each tube with water.

2. Disinfect completely by exposing the tubes, etc., to a temperature of 120° C. for twenty minutes (*vide* page 42).

(If an autoclave is not available, the tubes must be

placed in a digester, or even a large pan or pail with a tightly fitting cover, and boiled vigourously for some thirty to forty-five minutes to ensure disinfection.)

3. Whilst still hot, empty each tube in turn and roughly clean its interior with a stiff test-tube brush.

4. Place the tubes in a bucket or other convenient receptacle, fill with water and add a handful of sapon or other soap powder. See that the tubes are full and submerged.

5. Fix the bucket over a large Bunsen flame and boil for thirty minutes.

6. Cleanse the interior of the tubes with the aid of test-tube brushes, and rinse thoroughly in cold water.

7. Drain off the water and immerse tubes in a large jar containing water acidulated with 2 to 5 per cent. hydrochloric acid. Allow them to remain there for about fifteen minutes.

8. Remove from the acid jar, drain, rinse thoroughly in running water, then with distilled water.

9. Invert the tubes and allow them to drain completely.

Dry the tubes and polish the glass inside and out with a soft cloth, such as selvyt.

Infected flasks, plates, and capsules must be treated in a similar manner.

Flasks which have been used only in the preparation of media must be cleaned immediately they are finished with. Fill each flask with water to which some soap powder and a few crystals of potassium permanganate have been added, and let boil over the naked flame. The interior of the flask can then usually be perfectly cleaned with the aid of a flask brush, but in some cases water acidulated with 5 per cent. nitric acid, or a large wad of wet cotton-wool previously rolled in silver sand, must be shaken around the interior of the flask, after which rinse thoroughly with clean water, dry, and polish.

Infected Pipettes.—

1. Plunge infected pipettes immediately after use into tall glass cylinders containing a 2 per cent. solution of lysol, and allow them to remain therein for some days.

2. Remove from the jar and drain. Boil in water to which a little soap has been added, for thirty minutes.

3. Rinse thoroughly in cold water.

4. Immerse in 5 per cent. nitric acid for an hour or two.

5. Rinse again in running water to remove all traces of acid.

6. Complete the cleaning as described under "new pipettes."

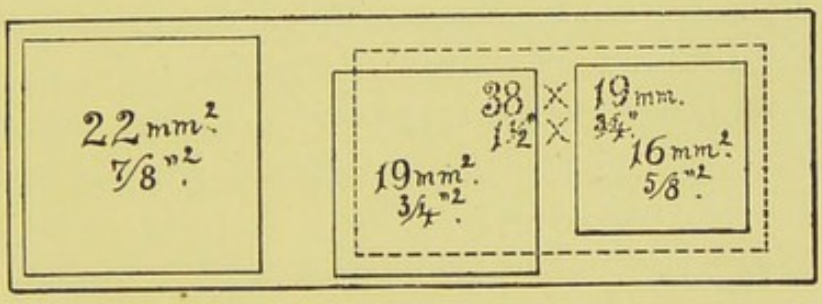


Fig. 15.—Slides and cover-slips, actual size.

Slides and **cover-slips** (Fig. 15), when first purchased, have "greasy" surfaces, upon which water gathers in minute drops and effectually prevents the spreading of thin, even films.

Microscopical Slides.—The slides in general use are those known as "three by one" slips (measuring 3 inches by 1 inch, or 76 by 26 mm.), and should be of good white crown glass, with ground edges.

New slides should be allowed to remain in alcohol acidulated with 5 per cent. hydrochloric acid for some hours, rinsed in running water, roughly drained on a towel, dried, and finally polished with a selvyt cloth.

Cover-slips.—The most useful sizes are the 19 mm.

squares for ordinary cover-glass film preparations, and 38 by 19 mm. rectangles for blood films and serial sections; both varieties must be of "No. 1" thickness, which varies between 0.15 and 0.22 mm., that they may be available for use with the high-power immersion lenses.

Cover-slips should be cleaned in the following manner:

1. Drop the cover-slips one by one into an enamelled iron pot or tall glass beaker, containing a 10 per cent. solution of chromic acid.

2. Heat over a Bunsen flame and allow the acid to boil gently for twenty minutes.

NOTE.—A few pieces of pipe-clay or pumice may be placed in the beaker to prevent the "spurting" of the chromic acid.

3. Turn the cover-slips out into a flat glass dish and wash in running water under the tap until all trace of yellow colour has disappeared. During the washing keep the cover-slips in motion by imparting a rotatory movement to the dish.

4. Wash in distilled water in a similar manner.

5. Wash in rectified spirit.

6. Transfer the cover-slips, by means of a pair of clean forceps, previously heated in the Bunsen flame to destroy any trace of grease, to a small beaker of absolute alcohol.

Drain off the alcohol and transfer the cover-slips, by means of the forceps, to a wide-mouthed glass pot, containing absolute alcohol, in which they are to be stored, and stopper tightly.

NOTE.—After once being placed in the chromic acid, the cover-slips must on no account be touched by the fingers.

Used Slides and Cover-slips.—Used slides with the mounted cover-slip preparations, and cover-slips used for hanging-drop mounts, should, when discarded, be

thrown into a pot containing a 2 per cent. solution of lysol.

After immersion therein for a week or so, even the cover-slips mounted with Canada balsam can be readily detached from their slides.

Slides.—

1. Wash the slides thoroughly in running water.
2. Boil the slides in water to which "sapon" has been added, for half an hour.
3. Rinse thoroughly in cold water.
4. Dry and polish with a dry cloth.

Cover-slips.—

1. Wash the cover-slips thoroughly in running water.
2. Boil the cover-slips in 10 per cent. solution of chromic acid, as for new cover-slips.
3. Wash thoroughly in running water.
4. Pick out those cover-slips which show much adherent dirty matter, and rub them between thumb and forefinger under the water tap. The dirt usually rubs off easily, as it has become friable from contact with the chromic acid.
5. Return all the cover-slips to the beaker, fill in *fresh* chromic acid solution, and treat as new cover-slips.

NOTE.—*Test-tubes*, *plates*, *capsules*, etc., which, from long use, have become scratched and hazy, or which cannot be cleaned in any other way, may be dealt with by immersing them in an enamelled iron bath, containing water acidulated with hydrofluoric acid 1 per cent., for ten minutes, rinsing thoroughly in water, drying, and polishing.

PLUGGING TEST-TUBES AND FLASKS.

Before sterilisation all test-tubes and flasks must be carefully plugged with cotton-wool, and for this purpose best absorbent cotton-wool (preferably that put up in cylindrical one-pound packets and inter-

leaved with tissue paper—known as surgeons' wool) should be employed.

1. For a test-tube or a small flask, tear a strip of cotton-wool some 10 cm. long by 2 cm. wide from the roll.

2. Turn in the ends neatly and roll the strip of wool lightly between the thumb and fingers of both hands to form a long cylinder.

3. Double this at the centre and introduce the now rounded end into the open mouth of the tube or flask.

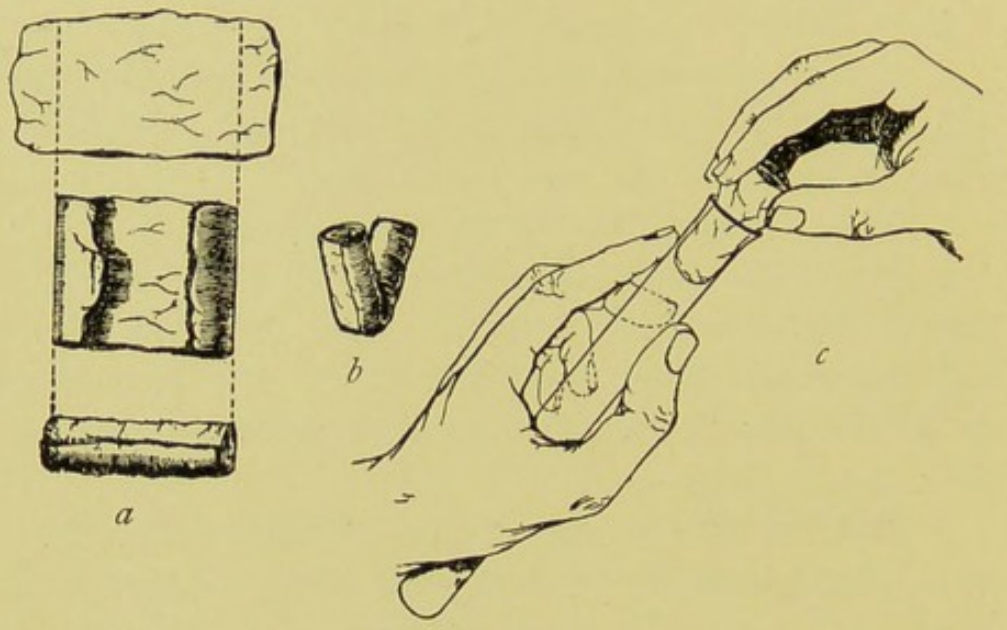


Fig. 16.—Plugging test-tubes: *a*, Cylinder of wool being rolled; *b*, cylinder of wool being doubled; *c*, cylinder of wool being inserted in tube.

4. Now, whilst supporting the wool between the thumb and fingers of the right hand, rotate the test-tube between those of the left, and gradually screw the plug of wool into its mouth for a distance of about 2.5 cm., leaving about the same length of wool projecting.

The plug must be firm and fit the tube or flask fairly tightly, sufficiently tightly in fact to bear the weight of the glass plus the amount of medium the vessel is intended to contain, but not so tightly as to prevent it from being easily removed by a screwing motion when

grasped between the fourth, or third and fourth fingers, and the palm of the hand.

For a large flask a similar but larger strip of wool must be taken; the method of making and inserting the plug is identical

II. METHODS OF STERILISATION.

STERILISING AGENTS.

STERILISATION—*i. e.*, the removal or the destruction of germ life—may be effected by the aid of various agents. As applied to the requirements of the bacteriological laboratory, many of these agents, such as electricity, sunlight, etc., are practically useless; whilst others are limited in their applications, or are so well suited to particular purposes as to be almost entirely restricted to such.

The sterilising agents in common use are:

Chemical Reagents.—*Disinfectants* (for the disinfection of glass and metal apparatus and of morbid tissues).

Heat.—(*a*) *Dry Heat:*

1. Naked flame (for the sterilisation of platinum needles, etc.).

2. Muffle furnace (for the sterilisation of filter candles, and for the destruction of morbid tissues).

3. Hot air (for the sterilisation of all glassware and of metallic substances).

(*b*) *Moist Heat:*

1. Water at 56° C. (for the sterilisation of certain albuminous fluids).

2. Water at 100° C. (for the sterilisation of surgical instruments, rubber tubing, and stoppers, etc.).

3. Streaming steam at 100° C. (for the sterilisation of media).

4. Superheated steam at 115° C. or 120° C. (for the disinfection of contaminated articles and old cultivations of bacteria).

Filters.—

1. Cotton-wool filters (for the sterilisation of air and gases).

2. Porcelain filters (for the sterilisation of various liquids).

METHODS OF APPLICATION.

Chemical Reagents, such as belong to the class known as antiseptics (*i. e.*, substances which inhibit the growth of, but do not destroy, bacterial life), are obviously useless. Disinfectants or germicides (*i. e.*, substances which destroy bacterial life), on the other hand, are of value in the disinfection of morbid material, and also of various pieces of apparatus, such as pipettes, pending their cleansing and complete sterilisation by other processes. To this class belong:

1. Lysol, 2 per cent. solution;
2. Perchloride of mercury, 0.1 per cent. solution;
3. Carbolic acid, 5 per cent. solution;
4. Absolute alcohol;
5. Ether;
6. Chloroform;
7. Volatile oils, such as oil of mustard, oil of garlic, arranged in order of general utility. Formaldehyde is perhaps a more powerful germicide than any of the above, but its penetrating vapor restricts its use. These disinfectants are but little used in the final sterilisation of apparatus, chiefly on account of the difficulty of completely removing such substances, for even traces of these chemicals are sufficient to so inhibit or alter the growth of bacteria brought into contact with them, as to vitiate subsequent experiments conducted by the aid of apparatus sterilised in this manner.

NOTE.—Tubes, flasks, filter flasks, pipettes, glass tubing, etc., may be rapidly sterilised, in case of emergency, by washing, in turn, with distilled water, perchloride of mercury solution, alcohol, and ether, drain-

ing, and finally gently heating over a gas flame to completely drive off the ether vapor. Chloroform or volatile oils may be added to various fluids in order to effect the destruction of contained bacteria, and when this has been done, may be completely driven off from the fluid by the application of gentle heat.

Dry Heat.—The *naked flame* of the Bunsen burner is used for sterilising the platinum needles, the points of forceps, or other small instruments, cover-glasses, etc., a very short exposure to this heat being sufficient to ensure sterilisation.

Muffle Furnace (Fig. 17).—This form of heat is chiefly used for the destruction of the dead bodies of small infected animals, morbid tissues, etc., but is also employed for the sterilisation of porcelain filter candles.

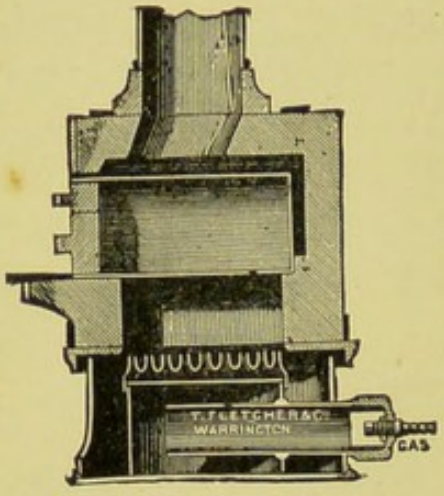


Fig. 17.—Muffle furnace.

Filter candles are disinfected immediately after use by boiling in a beaker of water for some fifteen or twenty minutes. This treatment, however, leaves the dead bodies of the bacteria upon the surface and blocking the interstices of the filter.

To destroy the organic matter and prepare the filter candle for further use proceed as follows:

1. Roll each bougie up in a piece of asbestos cloth, secure the ends with a few turns of copper wire, and place inside the muffle (a small muffle 76 by 88 by 163 mm. will hold perhaps four small Berkfeld candles).

2. Light the gas and raise the contents of the muffle to a white heat; maintain this temperature for five minutes.

3. Extinguish the gas, and when the muffle and its

contents have become quite cold, remove the filter candles from the interior of the muffle, and store without removing the asbestos wrappings, in sterile metal boxes.

Hot Air.—Hot air at 150° C. destroys all bacteria, spores, etc., in about thirty minutes, whilst a few minutes' exposure to a temperature of 170° to 180° C. will effect the same result. This method is only applicable to glass and metallic substances, and the small bulk of cotton-wool comprised in the test-tube plugs, etc. Large masses of fabric are not effectually sterilised by dry heat, as its power of penetration is not great.

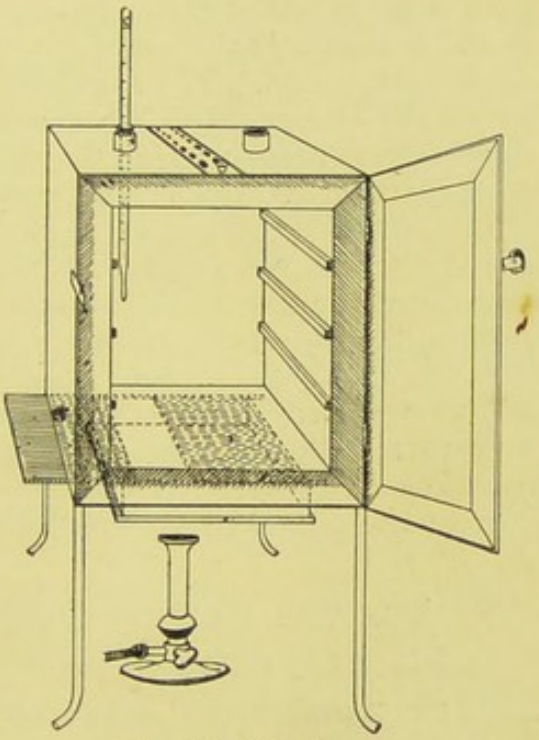


Fig. 18.—Hot-air oven.

Sterilisation by hot air is effected in the hot-air oven (Fig. 18). This is a rectangular, double-walled metal box, mounted on a stand and heated from below by a large Bunsen burner. One of the sides is hinged to form a door. The central portion of the metal bottom, on which the Bunsen flame would play, is cut away, and replaced by firebrick plates, which slide in metal grooves and are easily replaced when broken or worn out. The top of the oven is provided with a perforated ventilator slide and two tubulures, the one for the reception of a centigrade thermometer graduated to 200° or 250° C., the other for a thermo-regulator. The thermo-regulator is by no means a necessity, and it is more convenient to replace it by a

large bore thermometer with a sliding platinum point, connected with an electric bell, which can be easily adjusted to ring at any given temperature. The interior of the oven is provided with loose shelves upon which the articles to be sterilised are arranged, either singly or packed in square wire baskets or crates, kept specially for this purpose.

To prepare crates for the reception of test-tubes, etc., cover the bottom with a layer of thick asbestos cloth; or take some asbestos fibre, moisten it with a little water and knead it into a paste; plaster the paste over the bottom of each crate, working it into meshes and smoothing its surface by means of a pestle. When several crates have been thus treated, place them inside the hot-air oven, close the door, open the ventilating slide, light the gas, and run the temperature of the interior up to 160° C. After an interval of about ten minutes extinguish the gas, open the oven door, and allow the contents to cool. The asbestos now forms a smooth, dry, spongy layer over the bottom, which will last many months before needing renewal, and will considerably diminish the loss of tubes from breakage.

Copper cylinders and large test-tubes intended for the reception of pipettes are prepared in a similar manner, to protect the points of these articles.

To Use the Hot-air Oven.—

1. Place the crates of test-tubes, plates and pipettes in their metal cases, loose apparatus, etc., inside the oven, taking particular care that none of the cotton-wool plugs are in contact with the walls of the oven, otherwise the heat transmitted by the metal will char or even flame them.

2. Close the oven door, set the electric alarm to ring at 100° C., light the gas below, and open the ventilating slide, in order that any moisture left in the tubes, etc., may escape.

3. When the temperature of the oven has reached 100° C., close the ventilating slide; reset the alarm to ring at 175° C.

4. Run the temperature up to 175° C.

5. Extinguish the gas at once, and allow the apparatus to cool.

6. When the temperature of the interior, as recorded by the thermometer, has fallen to 60° C.,—*but not before*,—the door may be opened and the sterile articles removed and stored away.

N. B.—Neglect of this precautionary cooling of the oven to 60° C. will result in numerous cracked and broken tubes.

On removal from the oven, the cotton-wool plugs will probably be slightly brown in colour.

Metal instruments, such as knives, scissors, and forceps, may be sterilised in the hot-air oven as described above, but exposure to 175° C. is likely to seriously affect the temper of the steel and certainly blunts the cutting edges. If, however, it is desired to sterilise surgical instruments by hot air, they should be packed in a metal box, or boxes, and heated to 130° C. and retained at that temperature for about thirty minutes.

Moist Heat.—*Water at 56° C.*—This temperature, if maintained for thirty minutes, is sufficient to destroy the vegetative forms of bacteria, but has practically no effect on spores. Its use is limited to the sterilisation of such albuminous "fluid" media as would coagulate at a higher temperature.

METHOD.—

1. Fit up a water-bath, heated by a Bunsen flame which is controlled by a thermo-regulator, so that the temperature of the water remains at 56° C.

2. Immerse the tubes or flasks containing the albuminous fluid in the water-bath so that the upper level of such fluid is at least 2 cm. below the level of the water. (The temperature of the bath will now fall somewhat, but after a few minutes will again rise to 56° C.)

3. After thirty minutes' exposure to 56° C., extinguish the gas, remove the tubes or flasks from the bath, and subject them to the action of running water so that their contents are rapidly cooled.

4. The vegetative forms of bacteria present in the liquid being killed, stand it for twenty-four hours in a cool, dark place; at the end of that time some at least of the spores will have germinated and assumed the vegetative form.

5. Destroy these new vegetative forms by a similar exposure to 56° C. on the second day, whilst others, of slower germination, may be caught on the third day, and so on.

6. In order to ensure thorough sterilisation, repeat the process on each of six successive days.

This method of exposing liquids to a temperature of 56° C. in a water-bath for half an hour on each of six successive days is termed *fractional sterilisation.*

Water at 100° C. destroys the vegetative forms of bacteria almost instantaneously, and spores in from five to fifteen minutes. This method of sterilisation is applicable to the metal instruments, such as knives, forceps, etc., used in animal experiments, syringes, rubber corks, rubber and glass tubing, and other small apparatus, and is effected in what is usually spoken of as the "water steriliser."

This is a rectangular copper box, 26 cm. long, 18 cm. wide, and 12 cm. deep, mounted on legs, heated from below by a Bunsen or radial gas burner, and containing a movable copper wire tray, 2 cm. smaller in every dimension than the steriliser itself, and which is provided with handles. The top of the steriliser is hinged to form a lid.

METHOD.—

1. Place the instruments, etc., to be sterilised inside the copper basket, and replace the basket in the steriliser.

2. Pour a sufficient quantity of water into the steriliser, shut down the lid, and light the gas below.

3. After the water has boiled and steam has been issuing from beneath the lid for ten minutes, extinguish the gas, open the lid, and lift out the wire basket by its handles; the contained instruments, etc., are now sterile and ready for use.

4. After use, or when accidentally contaminated, replace the instruments in the basket and return that

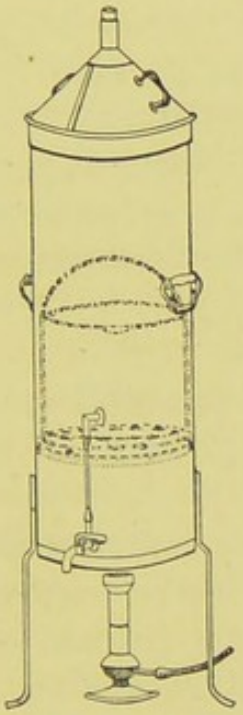


Fig. 19.—Koch's steriliser.

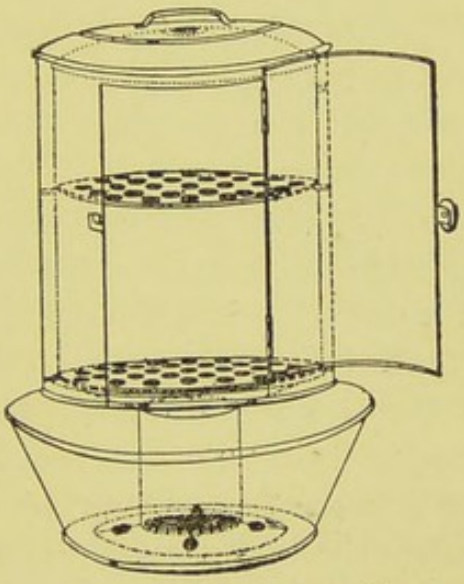


Fig. 20.—Arnold's steriliser.

to the steriliser; completely disinfect by a further boiling for fifteen minutes.

5. After disinfection, and whilst still hot, take out the instruments, carefully dry them at once, and return them to their store cases.

Streaming steam—i. e., steam at 100° C.—destroys the vegetative forms of bacteria in from fifteen to twenty minutes, and the sporing forms in from one to two hours. This method is chiefly used for the sterilisation of the various nutrient media intended for

the cultivation of bacteria, and is carried out in a steam kettle of special construction, known as Koch's steam steriliser (Fig. 19) or in one of its many modifications, the most efficient of which is Arnold's (Fig. 20).

The steam steriliser in its simplest form consists of a tall tinned-iron or copper cylindrical vessel, divided into two unequal parts by a movable perforated metal diaphragm, the lower, smaller portion serving for a water reservoir, and the upper for the reception of wire baskets containing the articles needing sterilisation. The vessel is closed by a loose conical lid, provided with handles, and perforated at its apex by a tubulure; it is mounted on a tripod stand and heated from below by a Bunsen burner. The more elaborate steriliser is cased with felt or asbestos board, and provided with a water gauge, also a tap for emptying the water compartment.

To Use the Steam Steriliser.—

1. Fill the water compartment to the level of the perforated diaphragm, place the lid in position, and light the Bunsen burner.

2. After the water has boiled, allow sufficient time to elapse for steam to replace the air in the sterilising compartment, as shown by the steam issuing in a steady, continuous stream from the tubulure in the lid.

3. Remove the lid, quickly lower the wire basket containing media tubes, etc., into the sterilising compartment until it rests on the diaphragm, and replace the lid.

4. After an interval of twenty minutes in the case of fluid media, or thirty minutes in the case of solid media, take off the lid and remove the basket with its contents.

5. Now, but not before, extinguish the gas.

Note.—After removing tubes, flasks, etc., from the steam steriliser, they should be at once separated freely in order to prevent moisture condensing upon

the cotton-wool plugs and soaking through into the interior of the tubes.

This treatment will destroy any vegetative forms of bacteria; during the hours of cooling any spores present will germinate, and the young organism will be destroyed by repeating the process twenty-four hours later; a third sterilisation after a similar interval makes assurance doubly sure.

The method of sterilising by exposure to streaming steam at 100° C. for twenty minutes on each of three consecutive days is termed *discontinuous* or *intermittent sterilisation.*

Continuous sterilisation, or exposure to steam at 100° C. for a period of one or two hours, is not to be recommended.

Superheated steam—i. e., steam under pressure in sealed vessels at a temperature of 115° C.—will destroy both the vegetative and the sporing forms of bacteria within fifteen minutes; if the pressure is increased, and the temperature raised to 120° C., the same end is attained in ten minutes. This method was formerly employed for the sterilisation of media, but when it was realised that hydrolytic changes occurred in media subjected to this high temperature, which rendered them unfit for the cultivation of the more delicate micro-organisms, its use was restricted almost entirely to the disinfection of such contaminated articles, old cultivations, etc., as could not be dealt with by dry heat or the actual furnace. Sterilisation by means of superheated steam is carried out in a special boiler—Chamberland's autoclave (Fig. 21). The autoclave consists of a stout copper cylinder, provided with a copper or gun-metal lid, which is secured in place by means of bolts and thumbscrews, the joint between the cylinder and its lid being hermetically sealed by the interposition of a rubber washer. The cover is perforated for a branched tube carrying a vent cock, a manometer, and

a safety valve. The copper boiler is mounted in the upper half of a cylindrical sheet-iron case—two concentric circular rows of Bunsen burners, each circle having an independent gas-supply, occupying the lower half. In the interior of the boiler is a large movable wire basket, mounted on legs, for the reception of the articles to be sterilised.

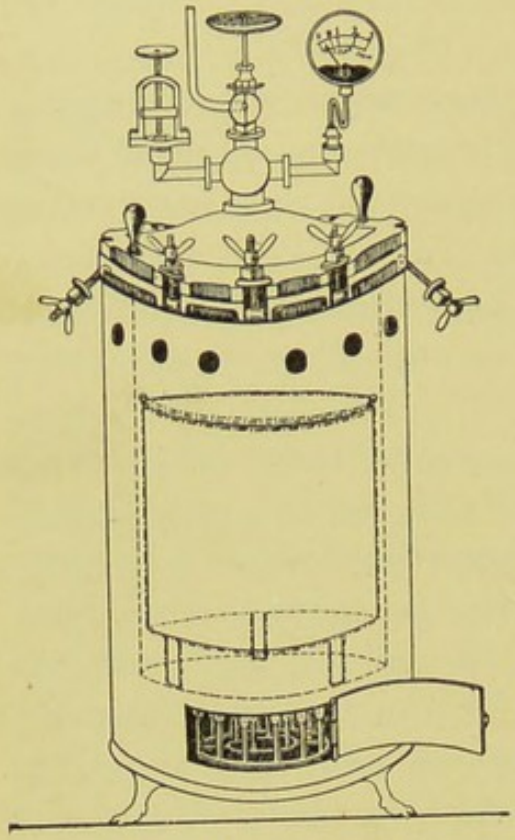


Fig. 21.—Autoclave.

To Use the Autoclave.—

1. Pack the articles to be sterilised in the wire basket.

2. Run water into the boiler to the level of the bottom of the basket; also fill the contained flasks and tubes with water.

3. See that the rubber washer is in position, then replace the cover and fasten it tightly on to the autoclave by means of the thumbscrews.

4. Open the vent cock and light both rings of burners.

5. When steam is issuing in a steady, continuous stream from the vent tube, shut off the vent cock and extinguish the outer ring of gas burners.

6. Wait until the index of the manometer records a temperature of 120° C., then regulate the gas and the spring safety valve in such a manner that this temperature is just maintained, and leave it thus for twenty minutes.

7. Extinguish the gas and allow the manometer index to fall to zero.

8. Now open the vent cock slowly, and allow the

internal pressure to adjust itself to that of the atmosphere.

9. Remove the cover and take out the sterilised contents.

Filters. — (*a*) *Cotton-wool.* — Practically the only method in use in the laboratory for the sterilisation of air or of a gas is by filtration through dry cotton-wool or glass-wool, the fibres of which entangle the micro-organisms and prevent their passage.

Perhaps the best example of such a filter is the cotton-wool plug which closes the mouth of a culture tube. Not only does ordinary diffusion take place through it, but if a tube plugged in the usual manner with cotton-wool is removed from the hot incubator, the temperature of the contained air rapidly falls to that of the

Fig. 22.—Air filter.

laboratory, and a partial vacuum is formed; air passes into the tube, through the cotton-wool plug, to restore the equilibrium, and, so long as the plug remains dry, in a germ-free condition. If, however, the plug becomes moist, either by absorption from the atmosphere, or from liquids coming into contact with it, micro-organisms (especially the mould fungi) commence to multiply, and the long thread forms rapidly penetrate the substance of the plug, gain access to and contaminate the interior of the tube.

If it is desired to sterilise gases before admission to a vessel containing a pure cultivation of a micro-organism, as, for instance, when forcing a current of oxygen over or through a broth cultivation of the

diphtheria bacillus, this can be readily effected as follows:

1. Take a length of glass tubing of, say, 1.5 cm. diameter, in the centre of which a bulb has been blown, fill the bulb with dry cotton-wool, wrap a layer of cotton-wool around each end of the tube, and secure in position with a turn of thread or string; then sterilise the piece of apparatus in the hot-air oven.

2. Prepare the cultivation in a Ruffer or Woodhead flask (Fig. 23) the inlet tube of which has a layer of cotton-wool wrapped round it and secured by

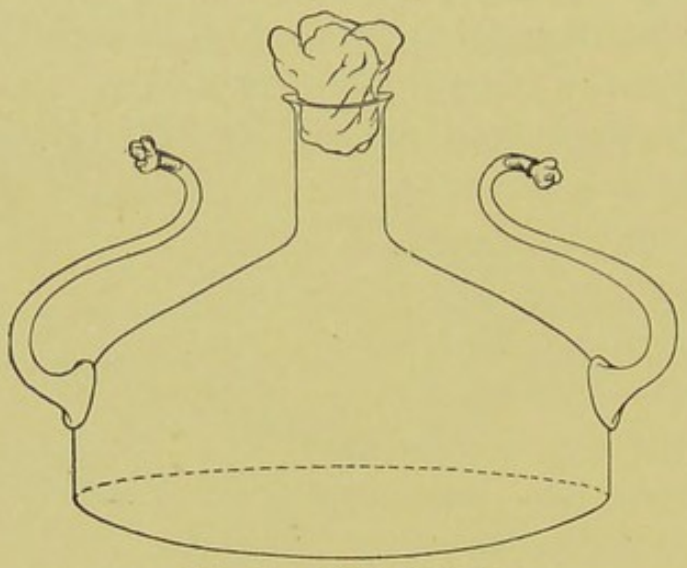


Fig. 23.—Ruffer's flask.

thread, whilst the exit tube is plugged in the usual manner.

3. Sterilise a short length of rubber tubing by boiling. Transfer it from the boiling water to a beaker of absolute alcohol.

4. Remove the rubber tube from the alcohol by means of a pair of forceps, drain it thoroughly, and pass through the flame of a Bunsen burner to burn off the last traces of alcohol.

5. Remove the cotton-wool wraps from the entry tube of the flask and from one end of the filter tube and rapidly couple them up by means of the sterile rubber tubing.

6. Connect the other end of the bulb tube with the delivery tube from the gas reservoir.

The gas in its passage through the dry sterile cotton-wool in the bulb of the filter tube will be freed from any contained micro-organisms and will enter the flask in a sterile condition.

(*b*) *Porcelain.*—The sterilisation of liquids by filtration is effected by passing them through a cylindrical vessel, closed at one end like a test-tube, and made either of porous "biscuit" porcelain, hard-burnt and unglazed (Chamberland system), or of Kieselguhr, a fine diatomaceous earth (Berkfeld system), and termed a "bougie" or "candle" (Fig. 24).

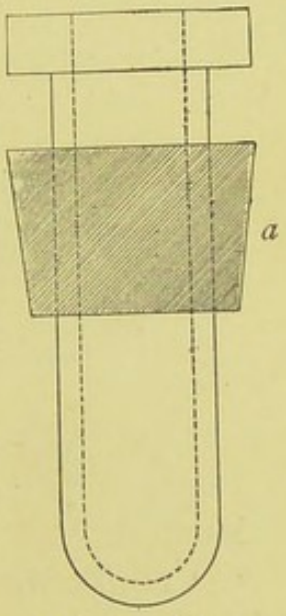


Fig. 24.—Filter candle.

In this method the bacteria are retained in the pores of the filter while the liquid passes through in a germ-free contdition.

It is obvious that to be effective the pores of the filter must be extremely minute, and therefore the rate of filtration will usually be slow. To overcome this defect, aspiration or pressure, or a combination of the two, may be employed to hasten the process.

Apparatus Required.—

1. Separatory funnel containing the unfiltered fluid.
2. Sterile filter candle, the open end fitted with a rubber stopper perforated to receive the delivery tube of the separatory funnel, and its neck passed through a large rubber washer (*a*) which fits the mouth of the filter flask.
3. Sterile filter flask of suitable size, for the reception of the filtered fluid, its mouth closed by a cotton-wool plug.
4. Water injector pump, or Geryk's pump (an air pump on the hydraulic principle, sealed by means of low vapor-tension oil, Fig. 25).

If this latter is employed, a Wulff's bottle, fitted as a wash-bottle and containing sulphuric acid, must be interposed between the filter flask and the pump, in order to prevent moist air reaching the oil in the pump.

5. Air filter (*vide* page 43) sterilised.
6. Pressure tubing.
7. Screw clamps (Fig. 26).

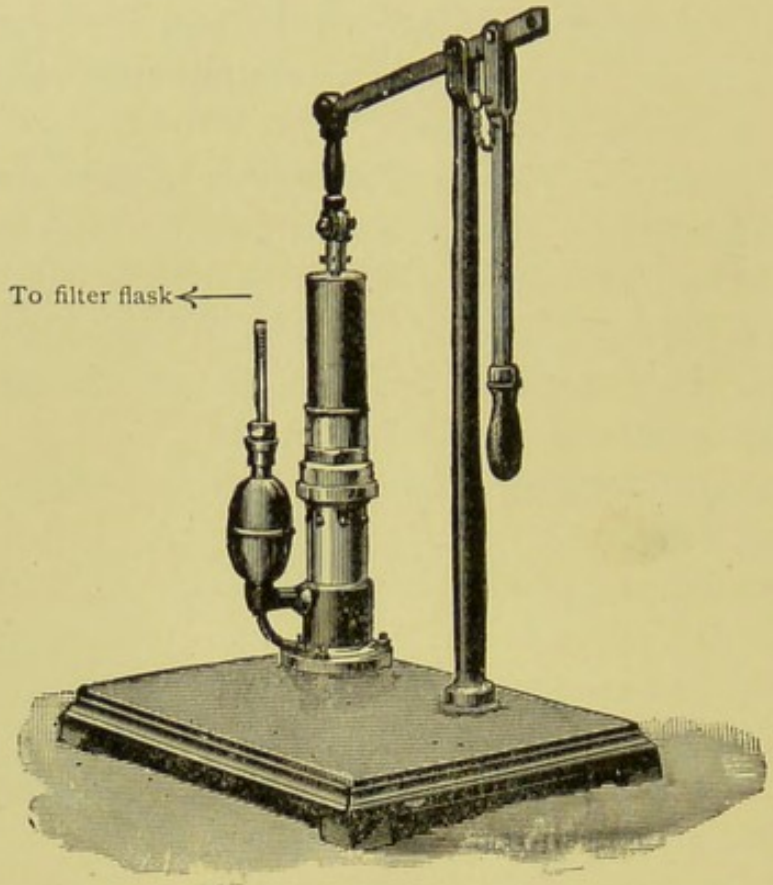


Fig. 25.—Geryk air pump.

METHOD.—

1. Couple the exhaust pipe of the suction pump with the lateral tube of the filter flask (first removing the cotton-wool plug from this latter), by means of pressure tubing, interposing, if necessary, the wash-bottle of acid.

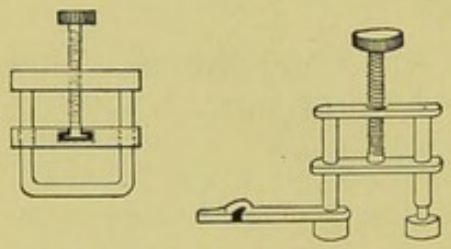


Fig. 26.—Screw clamps.

2. Remove the cotton-wool plug and adjust the porcelain candle in the neck of the filter flask.

3. Attach the nozzle of the separatory funnel to the filter candle by means of the perforated rubber stopper (Fig. 27).

4. Open the tap of the funnel, and exhaust the air from the filter flask and wash-bottle; maintain the vacuum until the filtration is complete.

5. Adjust a screw clamp to the pressure tubing attached to the lateral branch of the filter flask; screw it up tightly, and disconnect the acid wash-bottle.

6. Attach the air filter to the open end of the pressure tubing; open the screw clamp gradually, and allow

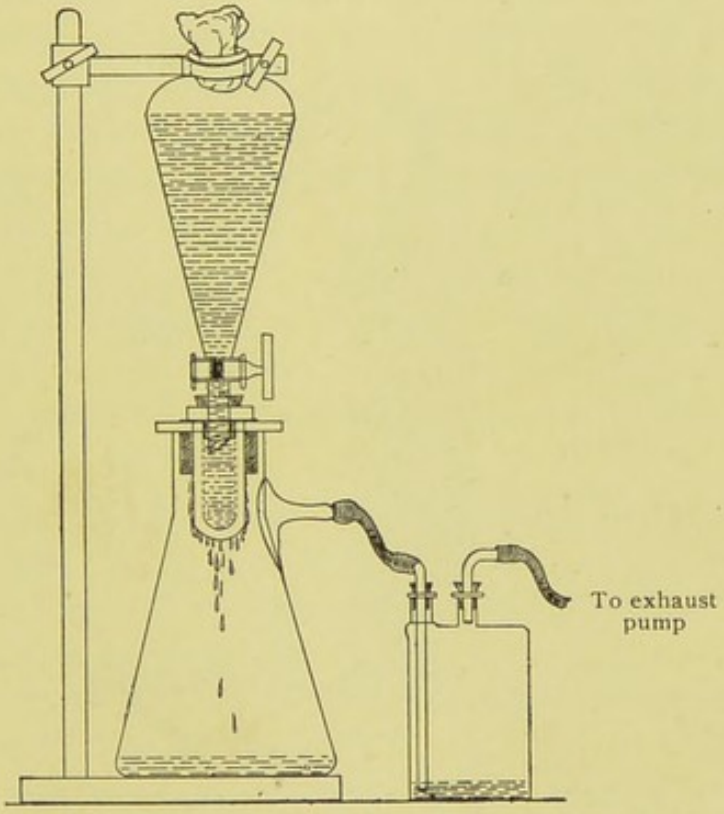


Fig. 27.—Apparatus arranged for filtering—aspiration.

filtered air to enter the flask, to destroy the negative pressure.

7. Detach the rubber tubing from the lateral branch of the flask, flame the end of the branch in the Bunsen, and plug its orifice with sterile cotton-wool.

8. Remove the filter candle from the mouth of the flask, flame the mouth, and plug with sterile cotton-wool.

9. Disinfect the filter candle and separatory funnel by boiling.

If it is found necessary to employ pressure in addition to or in place of suction, insert a perforated rubber stopper into the mouth of the separatory funnel and secure in position with copper wire; next fit a piece of glass tubing through the stopper, and connect the external orifice with an air-pressure pump of some kind (an ordinary foot pump such as is employed for inflating bicycle tires is one of the most generally use-

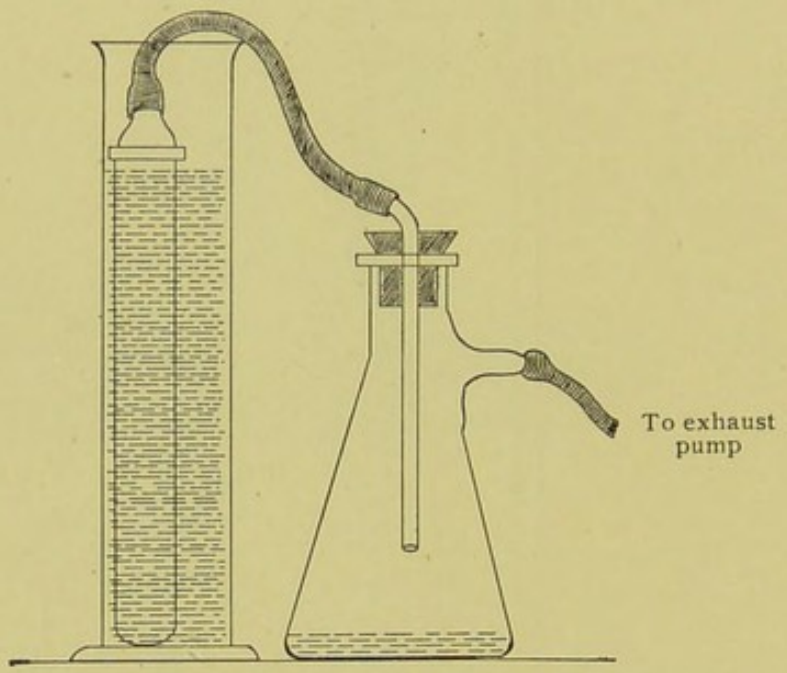


Fig. 28.—Apparatus arranged for filtering.

ful, for this purpose) or with a cylinder of compressed air or other gas.

Some forms of filter candle are made with the open end contracted into a delivery nozzle, which is glazed. In this case the apparatus is fitted up in a slightly different manner; the fluid to be filtered is contained in an open cylinder into which the candle is plunged, while its delivery nozzle is connected with the filter flask by means of a piece of flexible pressure tubing (previously sterilised by boiling), as in figure 28.

In order to filter a large bulk of fluid very rapidly it is necessary to use a higher pressure than glass would stand, and in these cases the metal receptacle designed by Pakes, to hold the filter candle itself as well as the fluid to be filtered, should be employed. (A vacuum should also be maintained in the filter flask, by means of an exhaust pump, during the entire process.)

This piece of apparatus consists of a brass cylinder, capacity 2500 c.c., with two shoulders; and an opening in the neck at each end, provided with screw threads.

A nut carrying a pressure gauge fits into the top screw; and into the bottom is fitted a brass cylinder carrying the filter candle and prolonged downwards into a delivery tube. Leakage is prevented by means of rubber washers.

Into the top shoulder a tube is inserted, bent at right angles and provided with a tap. All the brass-work is tinned inside (Fig. 29, *a*). In use the reservoir is generally mounted on a tripod stand.

To Sterilise.—

1. Insert the filter candle into its cylinder and screw this loosely on.

2. Wrap a layer of cotton-wool around the delivery tube and fasten in position.

3. Remove the nut carrying the pressure gauge and plug the neck with cotton-wool.

4. Heat the whole apparatus in the autoclave at 120° C. for twenty minutes.

METHOD.—

1. Remove the apparatus from the autoclave, and allow it to cool.

2. Screw home the box carrying the bougie.

3. Set the apparatus up in position, with its delivery tube (from which the cotton-wool wrapping has been removed) passing through a perforated rubber stopper in the neck of a filter flask.

4. Fill the fluid to be filtered into the cylinder and

screw on the nut carrying the pressure gauge. (This nut should be immersed in boiling water for a few minutes previous to screwing on, in order to sterilise it.)

5. Connect the horizontal arm of the entry tube with a cylinder of compressed oxygen (or carbon dioxide, Fig. 29, *b*), by means of pressure tubing.

6. Connect the lateral arm of the filter flask with

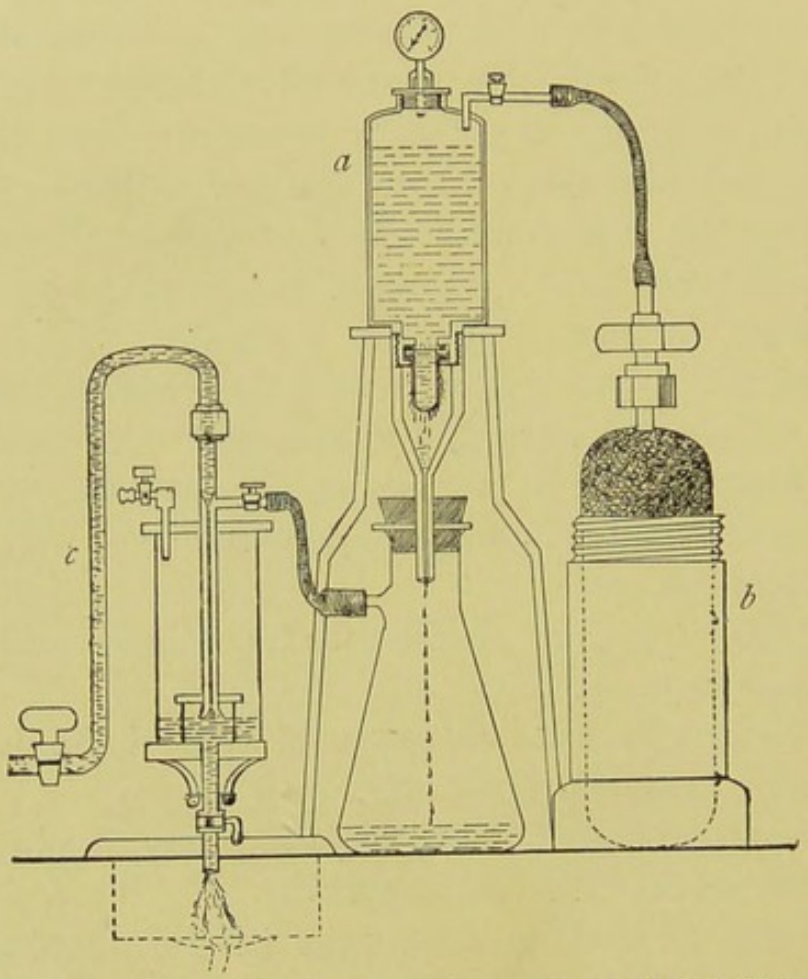


Fig. 29 —Pakes' filtering reservoir—pressure and aspiration.

the exhaust pump (Fig. 29, *c*) and start the latter working.

7. Open the tap of the gas cylinder; then open the tap on the entry tube of the filter cylinder and raise the pressure in its interior until the desired point is recorded on the manometer. Maintain this pressure until filtration is completed, by regulating the tap on the entry tube.

III. THE MICROSCOPE.

The essentials of a microscope for bacteriological work may be briefly summed up as follows:

The instrument of the monocular type must be of

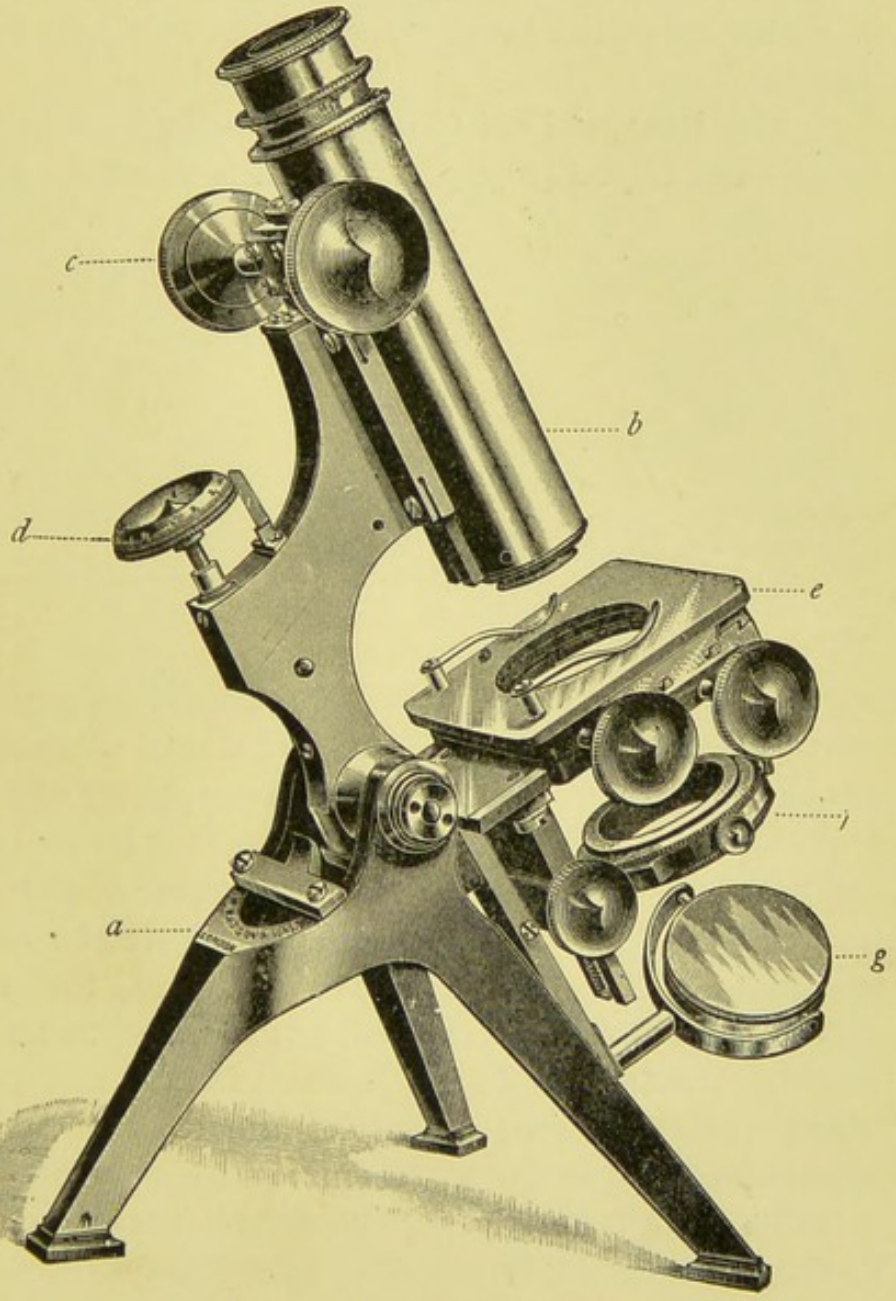


Fig. 30.—Microscope complete.

good workmanship and well finished, rigid, firm, and free from vibration, not only when upright, but also when inclined to an angle or in the horizontal position. The various joints and movements must work smoothly and precisely, equally free from the defects of "loss of time" and "slipping." All screws, etc., should conform to the Royal Microscopical Society's standard. It must also be provided with good lenses and a sufficiently large stage. The details of its component parts, to which attention must be specially directed, are as follows:

1. The Base or Foot (Fig. 30, *a*).—Two elementary forms—the tripod (Fig. 31, *a*) and the vertical column

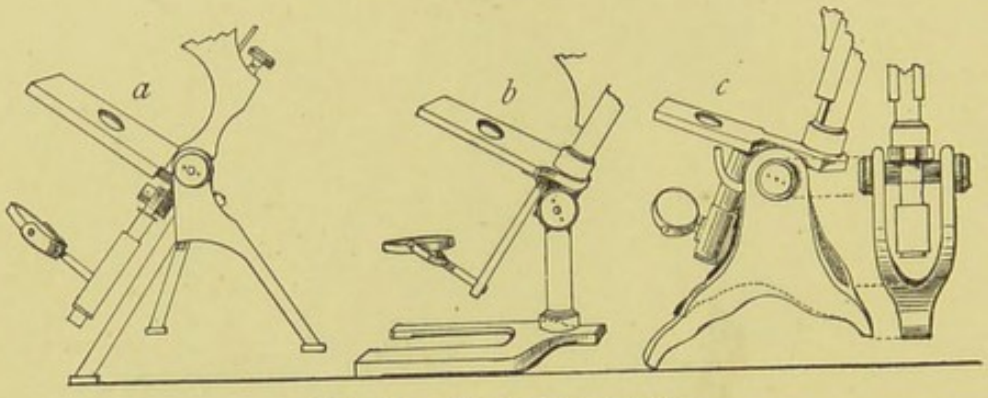


Fig. 31.—Foot, three types.

set into a plate (Fig. 31, *b*)—serve as the patterns for countless modifications in shape and size of this portion of the stand. The chief desiderata—stability and ease of manipulation—are attained in the first by means of the "spread" of the three feet, which are usually shod with cork; in the second, by the dead weight of the foot-plate. The tripod is mechanically the more correct form, and for practical use is much to be preferred. Its chief rival, the Jackson foot (Fig. 31, *c*), is based upon the same principle, and on the score of appearance has much to recommend it.

2. The body tube (Fig. 30, *b*) may be either that known as the "long" or "English" (length 250 mm.), or the "short" or "Continental" (length 160 mm.). Neither

length appears to possess any material advantage over the other, but it is absolutely necessary to secure objectives which have been manufactured for the particular tube length chosen. In the high-class microscope of the present day the body tube is usually shorter than the Continental, but is provided with a draw tube which, when fully extended, gives a tube length greater

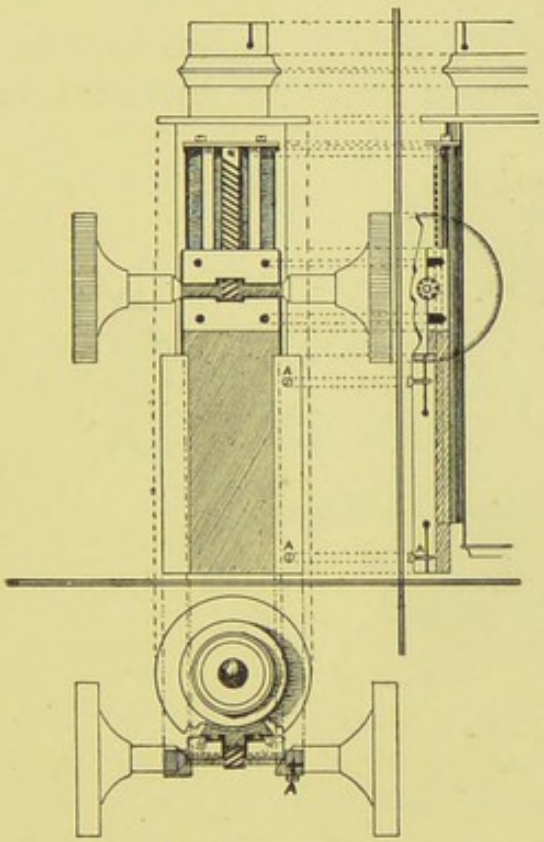


Fig. 32.—Coarse adjustment.

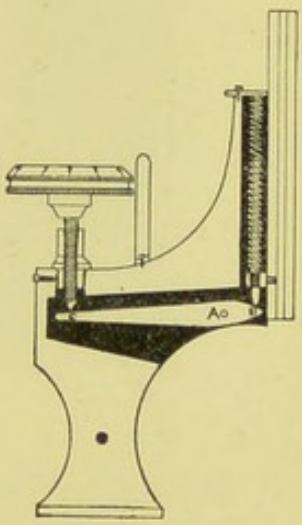


Fig. 33.—Fine adjustment.

than the English, thus permitting the use of either form of objective.

(Optical tube length = distance from the back lens of the objective to the field glass of the ocular.
Mechanical tube length = distance from the end of the nosepiece to the eyeglass of the ocular.)

3. The **coarse adjustment** (Fig. 30, *c*) should be a rack-and-pinion movement, steadiness and smoothness of action being secured by means of deeply bevelled edges and careful countersinking (Fig. 32).

4. The **fine adjustment** (Fig. 30, *d*) should on no account depend upon the action of springs, but should be of the lever pattern, preferably the Nelson (Fig. 33). In this form the unequal length of the arms of the lever secures very delicate movement, and, moreover, only a small portion of the weight of the body tube is transmitted to the thread of the vertical screw actuating the movement.

5. The **stage** (Fig. 30, *e*) should be square in shape and large in area,—at least 12 cm.,—flat and rigid, in

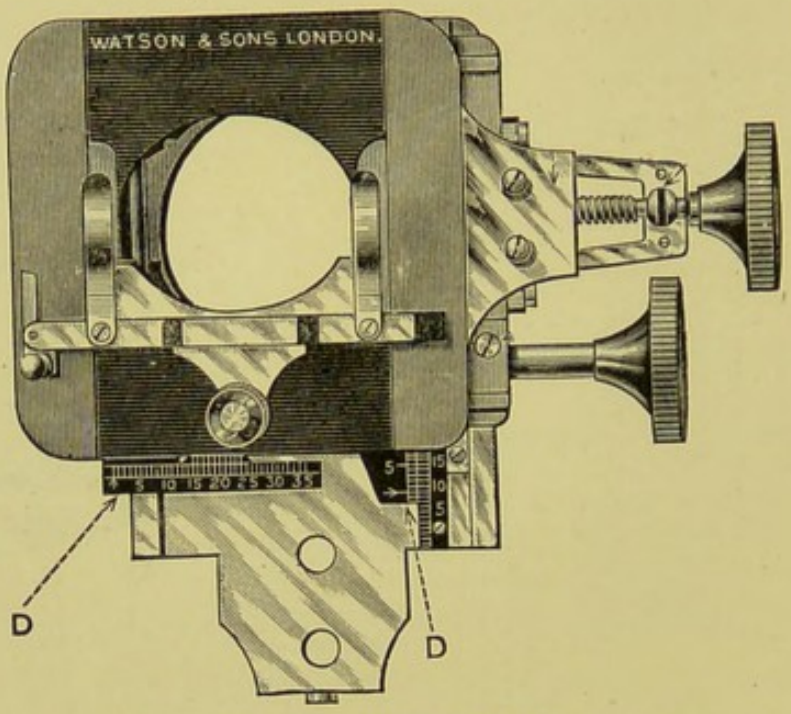


Fig. 34.—Mechanical stage.

order to afford a safe support for the Petri dish used for plate cultivations; and should be supplied with spring clips (removable at will) to secure the 3 by 1 glass slides.

A mechanical stage must be classed as a necessity rather than a luxury so far as the bacteriologist is concerned, as when working with high powers, and especially when examining hanging-drop specimens, it is almost impossible to execute sufficiently delicate movements with the fingers. In selecting a mechanical stage, preference should be given to one which forms

an integral part of the ordinary stage (Fig. 34) rather than one which needs to be clamped on every time it is required. The mechanical stage should be fitted with three (removable) screw studs, so that if necessary a Vernier finder (Fig. 34, D), such as is usually fitted to this class of stage, or a Maltwood finder, may be used.

6. Diaphragm.—Separate single diaphragms must be avoided; a revolving plate pierced with different-sized apertures and secured below the stage is preferable, but undoubtedly the best form is the "iris" diaphragm (Fig. 35).

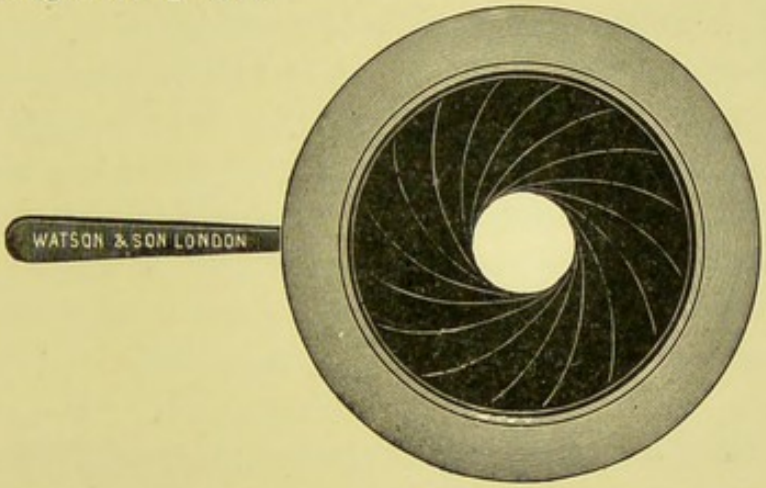


Fig. 35.—Diaphragm iris.

7. The **substage condenser** is a necessary part of the optical outfit. Its purpose is to collect the rays of light reflected by the mirror, by virtue of a short focus system of lenses, into a cone of large aperture (reducible at will by means of an iris diaphragm mounted as a part of the condenser), which can be accurately focussed on the plane of the object. This focussing must be performed anew for each object, on account of the variation in the thickness of the slides.

The form in most general use is that known as the Abbé (Fig. 36) and consists of a plano-convex lens mounted above a biconvex lens. This combination is carried in a screw-centering collar below the stage of

the microscope (Fig. 30, *f*), and must be accurately adjusted so that its optical axis coincides with that of the objective. Vertical movement of the entire substage apparatus effected by means of a rack and pinion is a decided advantage, and some means should be provided for temporarily removing the condenser from the optical axis of the microscope.

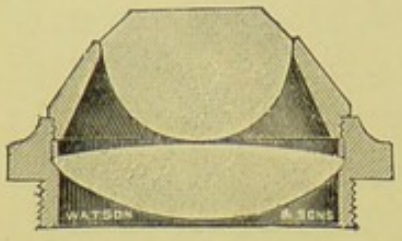


Fig. 36.—Optical part of Abbé illuminator.

8. Mirrors.—Below the substage condenser is attached a reversible circular frame bearing a plane mirror on one side and a concave mirror on the other (Fig. 30, *g*). The plane mirror is that usually employed, but occasionally, as for example when using low powers and with the condenser racked down and thrown out of the optical axis, the concave mirror is used.

9. Oculars, or Eyepieces.—Those known as the Huyghenian oculars (Fig. 37) will be sufficient for all ordinary work without resorting to the more expensive "compensation" oculars. Two or three, magnifying the "real" image (formed by the objective) four, six, or eight times respectively, form a useful equipment.

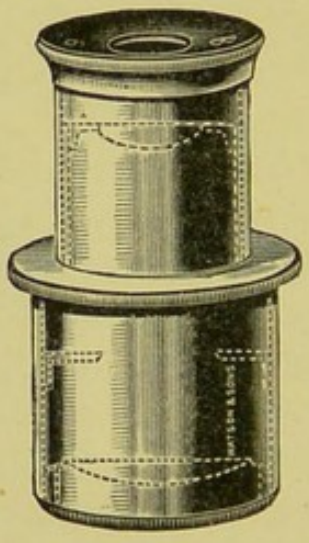


Fig. 37.—Huyghenian eyepiece.

10. Objectives. — Three objectives are necessary: one for low-power work—*e. g.*, 1 inch, $\frac{2}{3}$ inch, or $\frac{1}{2}$ inch; one for high-power work—*e. g.*, $\frac{1}{12}$ inch oil immersion lens; and an intermediate lens—*e. g.*, $\frac{1}{6}$ inch or $\frac{1}{8}$ inch (dry). These lenses must be carefully selected, especial attention being paid to the following points:

(*a*) *Correction of Spherical Aberration.*—Spherical

aberration gives rise to a distorted image, due to the central and peripheral rays focussing at different points.

(*b*) *Correction of Chromatic Aberration.*—Chromatic aberration gives rise to a coloured fringe around the edge of the field, which is due to the fact that the different-coloured rays of the spectrum possess varying refrangibilities and that a simple lens acts towards them as a prism.

(*c*) *Flatness of Field.*—The visual field should be large and, above all, *flat;* in other words, objects at the periphery of the field should be as distinctly "in focus" as those in the centre. Failing this, the lens should possess a large central "flat" area and the entire peripheral ring should come into focus at the same moment and with the least possible movement of the fine adjustment.

(*d*) *Good Definition.*—Actual magnification is, within limits, of course, of less value than clear definition and high resolving power, for it is upon these properties we depend for our knowledge of the detailed structure of the objects examined.

(*e*) *Numerical Aperture* (*N. A.*).—The numerical aperture may be defined, in general terms, as the ratio of the *effective* diameter of the back lens of the objective to its equivalent focal length. The determination of this point is a process requiring considerable technical skill and mathematical ability, and is completely beyond the powers of the average microscopist.[1]

Although with the increase in power it is increasingly difficult to combine all these corrections in one objective, they are brought to a high pitch of excellence in the present-day "achromatic" objectives, and so

[1] Its importance will be realised, however, when it is stated in the words of Professor Abbé: "The numerical aperture of a lens determines all its essential qualities; the brightness of the image increases with a given magnification and other things being equal, as the square of the aperture; the resolving and defining powers are directly related to it, the focal depth of differentiation of depths varies inversely as the aperture, and so forth."

remove the necessity for the use of the higher priced and less durable apochromatic lenses.

In selecting objectives the best "test" objects to employ are:

1. A thin (one cell layer), even "blood film," stained with eosin and counterstained with methylene-blue.

2. A thin cover-slip preparation of a young cultivation of the *B. diphtheriæ* (showing segmentation) stained with methylene-blue.

Accessories.—*Nosepiece.*—The first and most useful accessory is a nosepiece to carry two of the objectives (Fig. 38), or, better still, all three (Fig. 39). This nosepiece, preferably constructed of aluminium, is of the covered-in type, consisting of a curved plate

Fig. 38.—Double nosepiece.

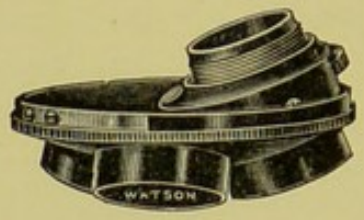


Fig. 39.—Triple nosepiece.

attached to the lower end of the body tube—a circular aperture being cut to correspond to the lumen of that tube. To the under surface of this plate is pivoted a similarly curved plate, fitted with three tubulures, each of which carries an objective. By rotating the lower plate each of the objectives can be brought successively in to the optical axis of the microscope.

Warm Stage (Fig. 40).—This is a flat metal case through the interior of which water of any required temperature can be circulated. It is made to clamp on to the stage of the microscope, and is perforated with a large hole coinciding with the optical axis of the microscope; and by raising the temperature of hanging-drop preparations, etc., placed upon it, above that of the surrounding atmosphere, is extremely useful

for observations on spore germination, hanging-drop cultivations, etc.

Eye Shade (Fig. 41).—This piece of apparatus consists of a pear-shaped piece of blackened metal, hinged

Fig. 40.—Warm stage.

to a collar which rotates on the upper part of the body tube of the microscope. It can be used to shut out the image of surrounding objects from the unoccupied eye, and when carrying out prolonged observations will be found of real service.

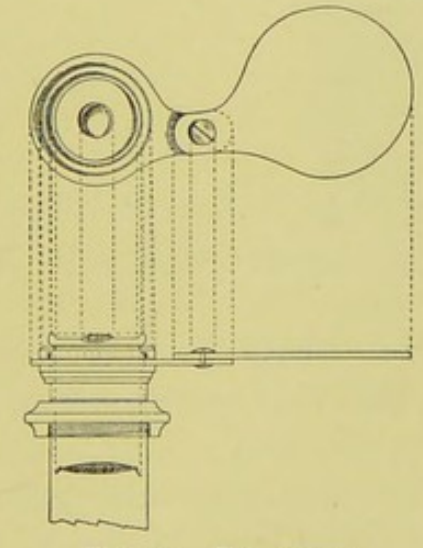


Fig. 41.—Eye screen.

Micrometer.—Some form of micrometer for the purpose of measuring bacteria and other objects is also essential. Details of those in general use will be found in the following pages.

METHODS OF MICROMETRY.

The unit of length as applied to the measurement of microscopical objects is the one-thousandth part of a millimetre (0.001 mm.), denominated a *micron* (sometimes, and erroneously, referred to as a micro-millimetre), and indicated in writing by the Greek letter μ. Of the many methods in use for the measurement of bacteria, three only will be here described, viz.:

By means of the stage micrometer.

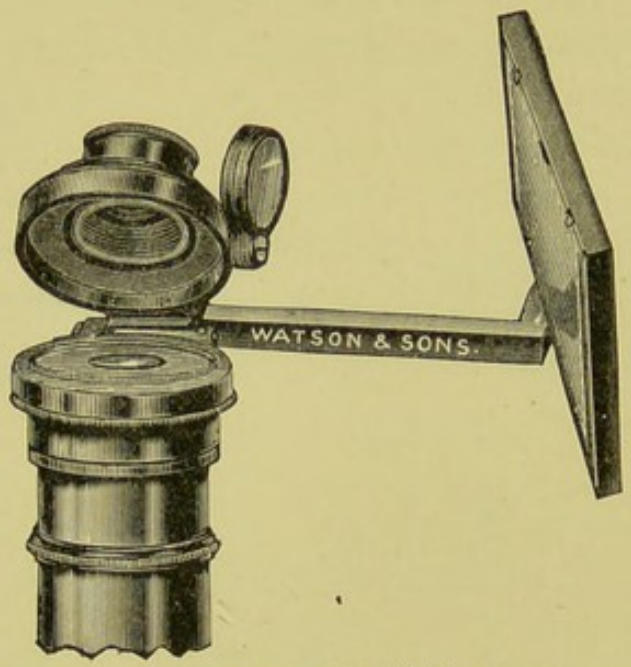


Fig. 42.—Camera lucida, Abbé pattern.

By means of the ocular or eyepiece micrometer.

By means of the filar micrometer (Ramsden's micrometer eyepiece).

(*a*) By means of the stage micrometer.

The **stage micrometer** is a 3 by 1 inch glass slip having engraved on it a scale divided to hundredths of a millimetre (0.01 mm.), every tenth line being made longer than the intervening ones, to facilitate counting. A cover-glass is cemented over the scale to protect it from injury.

1. Attach a camera lucida (of the Wollaston, Beale, or Abbé pattern) to the eyepiece of the microscope.

2. Adjust the micrometer on the stage of the microscope and accurately focus the divisions.

3. Project the scale of the stage micrometer on to a piece of paper and with pen or pencil sketch in the magnified image, each division of which corresponds to 10 μ. Mark on the paper the optical combination (ocular objective and tube length) employed to produce this particular magnification.

4. Repeat this procedure for each of the possible combinations of oculars and objectives fitted to the microscope supplied, and carefully preserve the scales thus obtained.

Fig. 43.—Eyepiece micrometer, ordinary.

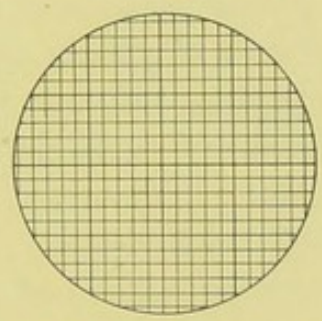


Fig. 44.—Eyepiece micrometer, net.

To measure an object by this method simply project the image on to the scale corresponding to the particular optical combination in use at the moment. Read off the number of divisions it occupies and express them as *micra*.

In place of preserving a scale for each optical combination, the object to be measured and the micrometer scale may be projected and sketched, in turn, on the same piece of paper.

(*b*) By means of the eyepiece micrometer.

The **eyepiece micrometer** is a circular glass disc having engraved on it a scale divided to tenths of a millimetre (0.1 mm.) (Fig. 43), or the entire surface ruled in 0.1

mm. squares (the net micrometer) (Fig. 44). It can be fitted inside the mount of any ocular just above the aperture of the diaphragm and must be adjusted exactly in the focus of the eyeglass.

Some makers mount the glass disc together with a circular cover-glass in such a way that when placed in position in any Huyghenian eyepiece of their own manufacture, the scale is exactly in focus.

The value of one division of the micrometer scale must first be ascertained for each optical combination by the aid of the stage micrometer, thus:

1. Insert the eyepiece micrometer inside the ocular and adjust the stage micrometer on the stage of the microscope.

2. Focus the scale of the stage micrometer accurately; the lines will appear to be immediately below those of the eyepiece micrometer. Make the lines on the two micrometers parallel by rotating the ocular.

3. Make two of the lines on the ocular micrometer coincide with those bounding one division of the stage micrometer; this is effected by increasing or diminishing the tube length; and note the number of included divisions.

4. Calculate the value of each division of the eyepiece micrometer in terms of μ, by means of the following formula:

$$x = 10\,y.$$

Where x = the number of included divisions of the eyepiece micrometer.

y = the number of included divisions of the stage micrometer.

5. Note the optical combination employed in this experiment and record it with the calculated micrometer value.

Repeat this process for each of the other combinations. Carefully record the results.

To measure an object by this method read off the

number of divisions of the eyepiece micrometer it occupies and express the result in micra by a reference to the standard value for the particular optical combination employed.

Zeiss prepares a compensating eyepiece micrometer for use with his apochromatic objectives, the divisions of which are so computed that (with a tube length of 160 mm.) the value of each is equivalent to as many micra as there are millimetres in the focal length of the objective employed.

(*c*) By means of the filar micrometer.

The **filar** or cobweb micrometer (Ramsden's microm-

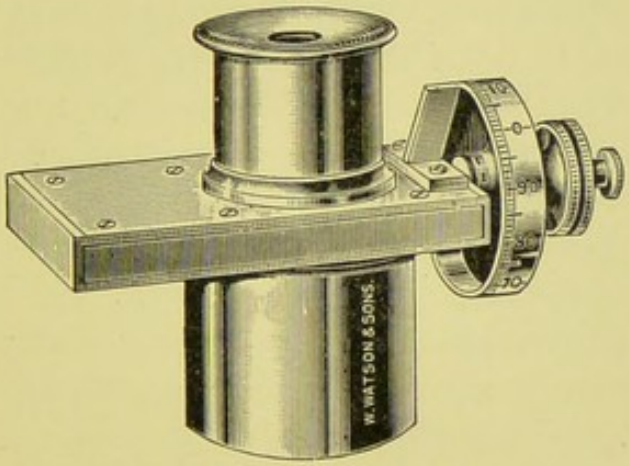


Fig. 45.—Ramsden's micrometer.

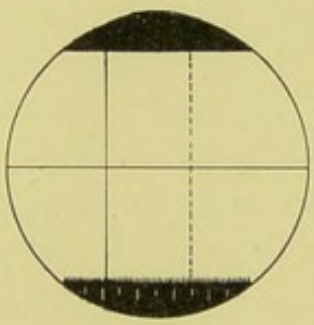


Fig. 46.—Ramsden's micrometer field.

eter eyepiece (Fig. 45) consists of an ocular having a fine "fixed" wire stretching horizontally across the field (Fig. 46), a vertical reference wire—fixed—adjusted at right angles to the first; and a fine wire, parallel to the reference wire, which can be moved across the field by the action of a micrometer screw; the trap head is divided into one hundred parts, which successively pass a fixed index as the head is turned. In the field is also fixed a comb with the intervals between its teeth corresponding to one complete revolution of this screw-head.

As in the previous method, the value of each division of the micrometer scale (*i. e.*, the comb) must first be determined for each optical combination. This is effected as follows:

1. Place the filar micrometer and the stage micrometer in their respective positions.

2. Rotate the screw of the filar micrometer until the movable wire coincides with the fixed one, and the index marks zero on the screw-head.

3. Focus the scale of each micrometer accurately, and make the lines on them parallel.

4. Rotate the head of the micrometer screw until the movable line has traversed one division of the stage micrometer. Note the number of complete revolutions (by means of the recording comb) and the fractions of a revolution (by means of scale on the head of the micrometer screw), which are required to measure the 0.01 mm.

5. Make several such estimations and average the results.

6. Note the optical combination employed in this experiment and record it carefully, together with the micrometer value in terms of μ.

7. Repeat this process for each of the different optical combinations and record the results.

To measure an object by this method, simply note the number of revolutions and fractions of a revolution of the screw-head required to traverse such object from edge to edge, and express the result as micra by reference to the recorded values for that particular optical combination.

IV. MICROSCOPICAL EXAMINATION OF BACTERIA AND OTHER MICRO-FUNGI.

APPARATUS AND REAGENTS USED IN ORDINARY MICROSCOPICAL EXAMINATION.

EACH student is provided with a set consisting of the following pieces of apparatus and reagents.

1. India-rubber "change-mat" upon which cover-glasses may be rested during the process of staining.

2. Squares of blotting paper about 10 cm., for drying cover-slips and slides.

(The filter paper known as "German lined"—a highly absorbent, closely woven paper, having an even surface and no loose "fluff" to adhere to the specimens —is the most useful for this purpose.)

3. Glass jar filled with 2 per cent. lysol solution for the reception of infected cover-glasses and useless slides.

4. Bunsen burner provided with by-pass.

5. Porcelain trough holding five or six hanging-drop slides (Fig. 47).

A hanging-drop slide is prepared by cementing a circular cell of tin, 13 to 15 mm. diameter, and 1 to 2 mm. in height, to the centre of a 3 by 1 slip by means of Canada balsam. It is often extremely convenient to have two of these cells cemented close together on one slide.

Another form of hanging-drop slide is made in which a circular or oval cell is ground out of the centre of a 3 by 1 slip. These are more expensive, less convenient to work with, and are more easily contaminated by drops of material under examination, and should be carefully avoided.

6. Three aluminium rods (Fig. 48), each about 25 cm. long and carrying a piece of 0.015 gauge platino-iridium wire 7.5 cm. in length. The end of one of the wires is bent round to form an oval loop, of about 1

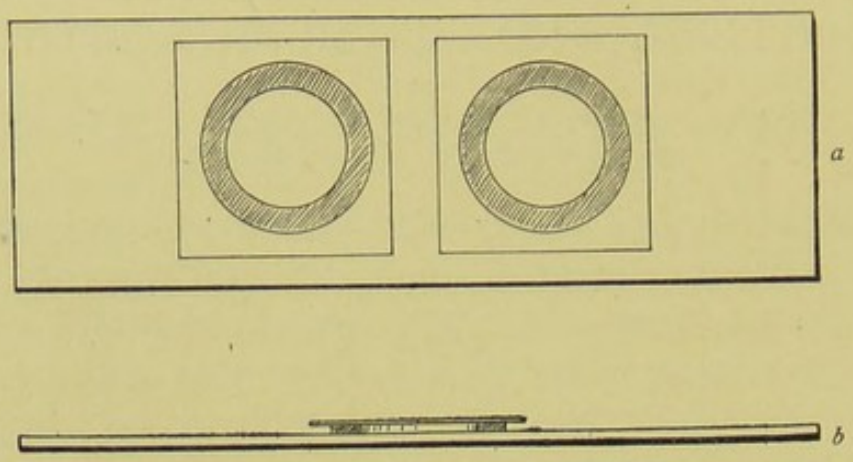


Fig. 47.—Hanging-drop slides: *a*, Double cell seen from above; *b*, single cell seen from the side.

mm. in its short diameter, and is termed a loop or an öse; the terminal 3 or 4 mm. of another wire is flattened out by hammering it on a smooth iron surface to form a "spatula"; the third is left untouched or is pointed by the aid of a file. These instruments are

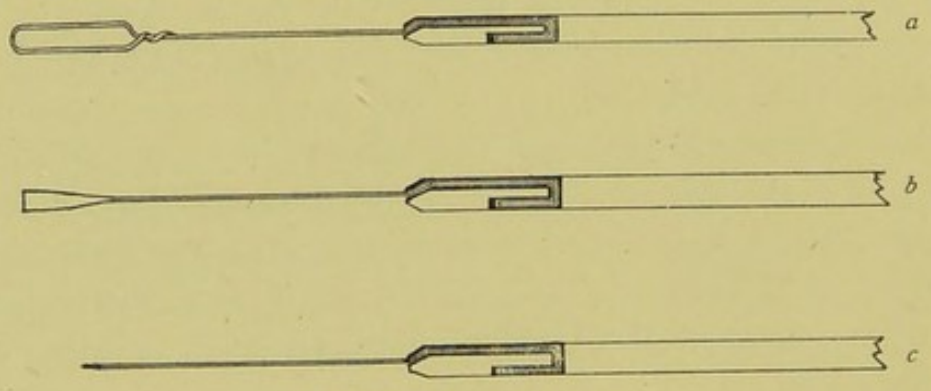


Fig. 48.—Ends of rods.

used for inoculating culture tubes and preparing specimens for microscopical examination.

The method of mounting these wires may be described as follows:

Take a piece of aluminium wire 25 cm. long and

about 0.25 cm. in diameter, and drill a fine hole completely through the wire about a centimetre from one end. Sink a straight narrow channel along one side of the wire, in its long axis, from the hole to the nearest end, shallow at first, but gradually becoming deeper.

On the opposite side of the wire make a short cut, 2 mm. in length, leading from the hole in the same direction.

Now pass one end of the platinum wire through the hole, turn up about 2 mm. at right angles and press the short piece into the short cut. Turn the long end of the wire sharply, also at right angles, and sink it into the long channel so that it emerges from about

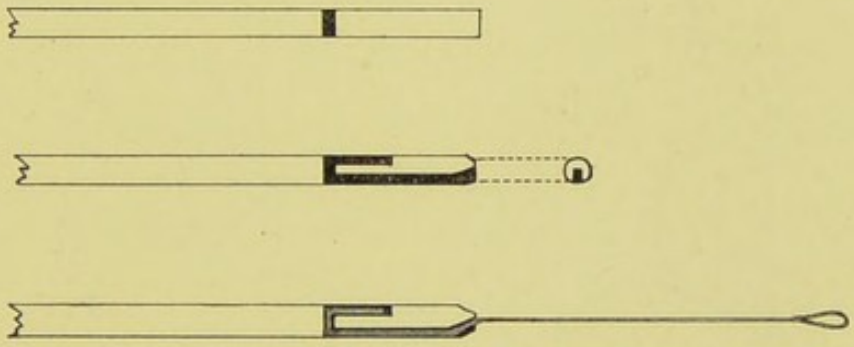


Fig. 49.—Platinum rod in aluminium handle—method of mounting.

the centre of the cut end of the aluminium wire (Fig. 49). A few sharp taps with a small hammer will now close in the side of the two channels over the wire and hold it securely.

The platinum wire may be fused into the end of a piece of glass rod, but such a handle is vastly inferior to aluminium and is not to be recommended.

7. Two pairs of sharp-pointed spring forceps (10 cm. long), one of which must be kept perfectly clean and reserved for handling clean cover-slips, the other being for use during staining operations.

8. A box of clean 3 by 1 glass slips.

9. A glass capsule with tightly fitting (ground on)

glass lid, containing clean cover-slips in absolute alcohol.

10. One of Faber's "grease pencils" (yellow, red, or blue) for writing on glass.

11. A wooden rack fitted with ten drop-bottles (Fig. 50) each 60 c.c. capacity, containing

Aniline water.
Gentian violet, saturated alcoholic solution.
Gram's iodine.
Absolute alcohol.

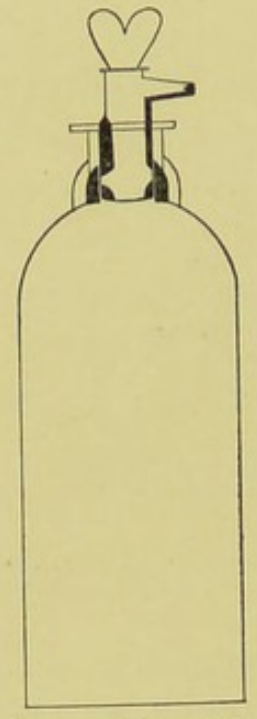


Fig. 50.—Drop-bottle.

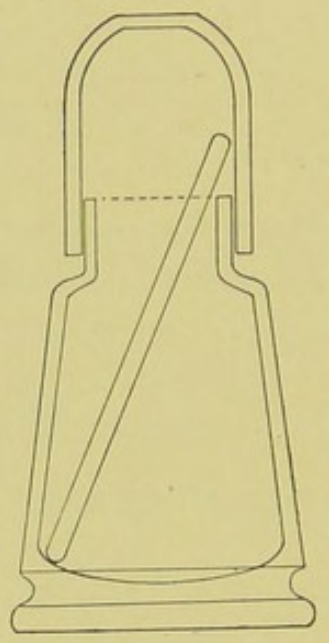


Fig. 51.—Canada balsam pot.

Eosin, yellowish, 1 per cent. aqueous solution.
Methylene-blue, Fuchsin, basic, } saturated alcoholic solution.
Carbolic acid, 5 per cent. aqueous solution.
Acetic acid, 1 per cent. solution.
Sulphuric acid, 25 per cent. solution.

And two pots with air-tight glass caps (Fig. 51), filled respectively with Canada balsam dissolved in xylol, and sterile vaseline, and each provided with a piece of glass rod.

METHODS OF EXAMINATION.

Bacteria, etc., are examined microscopically, both

1. In the living state, unstained, or stained.

2. After having been fixed, killed, and stained by suitable methods.

The preparation of a specimen from a tube cultivation for examination by these methods may be described as follows:

1. Living, Unstained.—(*a*) *"Fresh" Preparation.*—1. Clean and dry a 3 by 1 glass slip and place it on one of the squares of filter paper. Deposit a drop of water (preferably distilled) or a drop of 1 per cent. solution of caustic potash, on the centre of the slip, by means of the platinum loop.

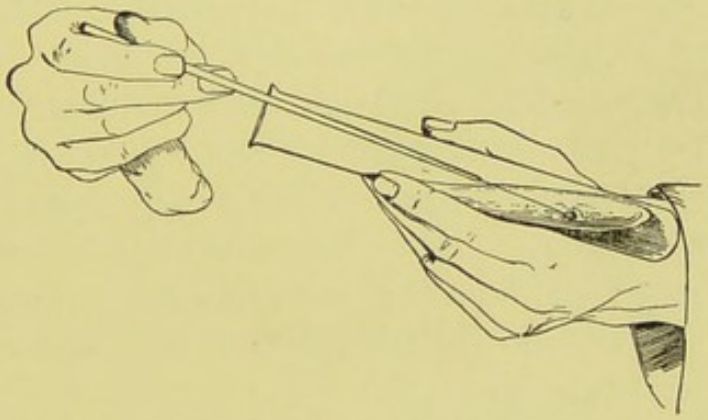


Fig. 52.—Holding tubes for removing cultivation, as seen from the front.

Technique of Opening and Closing a Culture Tube.

2. Take the tube cultivation in the left hand and ignite the cotton-wool plug by holding it to the flame of the Bunsen burner. Extinguish the flame by blowing on the plug, whilst rotating the tube on its long axis, its mouth directed vertically upwards, between the thumb and fingers. (This operation is termed "flaming the plug," and is intended to destroy any micro-organisms that may have become entangled in the loose fibres of the cotton-wool, and which, if not thus destroyed, might fall into the tube

when the plug is removed and so accidentally contaminate the cultivation.)

3. Hold the tube at or near its centre between the ends of the thumb and first two fingers of the left hand, and allow the sealed end to rest upon the back of the hand between the thumb and forefinger, the plug pointing to the right. Keep the tube as nearly in the horizontal position as is consistent with safety, to diminish the risk of the accidental entry of organisms (Fig. 52).

4. Take the handle of the loop between the thumb and forefinger of the right hand, holding the instrument in a position similar to that occupied by a pen or a paint-brush, and sterilise the platinum portion by holding it in the flame of a Bunsen burner until it is red hot. Sterilise the adjacent portion of the aluminium handle by passing it rapidly twice or thrice through the flame. After sterilising it, the loop must not be allowed to leave the hand or to touch against anything but the material it is intended to examine, until it is finished with and has been again sterilised.

5. Grasp the cotton-wool plug of the test-tube between the little finger and the palm of the right hand (whilst still holding the loop as directed in step 4), and remove it from the mouth of the tube by a "screwing" motion of the right hand.

6. Introduce the platinum loop into the tube and hold it in this position until satisfied that it is quite cool. (The cooling may be hastened by touching the loop on one of the drops of moisture which are usually to be found condensed on the interior of the glass tube, or by dipping it into the condensation water at the

Technique of Opening and Closing a Culture Tube.

bottom; at the same time care must be taken in the case of cultures of solid media to avoid touching either the medium or the growth.)

7. Remove a small portion of the growth by taking up a drop of liquid, in the case of a fluid culture, in the loop or by touching it on the surface of the growth when the culture is on solid medium; and withdraw the loop from the tube without again touching the medium or the glass sides of the tube.

8. Replace the cotton-wool plug in the mouth of the tube.

9. Mix the contents of the loop thoroughly with the drop of water on the 3 by 1 slide.

10. Again sterilise the loop as directed in step 4, and replace it in its stand.

11. Replace the tube cultivation in its rack or jar.

12. Remove a cover-slip from the glass capsule by means of the cover-slip forceps, rest it for a moment on its edge, on a piece of filter paper to remove the excess of alcohol, then pass it through the flame of the Bunsen burner. This burns off the remainder of the alcohol, and the cover-slip so "flamed" is now clean, dry, and sterile.

13. Lower the cover-slip, still held in the forceps, on to the surface of the drop of fluid on the 3 by 1 slip, carefully and gently, to avoid the inclusion of air bubbles.

14. Examine microscopically (*vide infra*).

During the microscopical examination, stains and other reagents may be run in under the cover-slips by the simple method of placing a drop of the reagent in contact with one edge of the cover-glass and applying the torn edge of a piece of blotting paper to the opposite side. The reagent may then be observed to flow across the field and come into contact with such of the micro-organisms as lie in its path.

(*b*) *Hanging-drop Preparation.*—

1. Smear a layer of sterile vaseline on the upper surface of the ring cell of a hanging-drop slide by means of the glass rod provided with the vaseline bottle, and place the slide on a piece of filter paper.

2. "Flame" a cover-slip and place it on the filter paper by the side of the hanging-drop slide.

3. Place a drop of water on the centre of the cover-slip by means of the platinum loop.

4. Obtain a small quantity of the material it is desired to examine, in the manner detailed above (steps 2 to 11 must be followed in their entirety and with the strictest exactitude whenever tube contents are being handled), and mix it with the drop of water on the cover-slip.

5. Raise the cover-slip in the points of the forceps and rapidly invert it on to the ring cell of the hanging-drop slide, so that the drop of fluid occupies the centre of the ring. (Carefully avoid contact between the drop of fluid and either the ring cell or the layer of vaseline. Should this happen, the now *infected* hanging-drop slide and its cover-slip must be dropped into the pot of lysol and a new preparation made.)

6. Press the cover-slip firmly down into the vaseline on to the top of the ring cell. (This spreads out the vaseline into a thin layer, and besides ensuring the adhesion of the cover-slip, seals the cells and so retards evaporation.)

7. Examine microscopically (*vide infra*).

Microscopical Examination of the Unstained Specimens.—

1. Place the body tube of the microscope in the vertical position.

2. Arrange the hanging-drop slide on the microscope stage so that the drop of fluid is in the optical axis of the instrument, and secure it in the position by means of the spring clips.

3. Use the $\frac{1}{6}$-inch objective, rack down the body tube until the front lens of the objective is almost in contact with the cover-slip—that is, well within its focal distance. This is best done whilst bending down the head to one side of the microscope, so that the eyes are on a level with the stage.

4. Apply the eye to the ocular and adjust the plane mirror to the position which secures the best illumination.

5. Rack the condenser down slightly and cut down the aperture of the iris diaphragm so that the light, although even, is dim.

6. Rack up the body tube by means of the coarse adjustment until the bacteria come into view; then focus exactly by means of the fine adjustment.

Some difficulty is often experienced at first in finding the hanging drop, and if the first attempt is unsuccessful, the student must not on any account, whilst still applying his eye to the ocular, rack the body tube down (for by so doing there is every likelihood of the front lens of the objective being forced through the cover-glass, and not only spoiling the specimen, but also contaminating the objective); but, on the contrary, withdraw his eye, rack the tube up, and commence again from step 2.

The examination of a "fresh" specimen or a "hanging-drop" preparation is directed to the determination of the following data:

1. The nature of the bacteria present—*e. g.*, cocci, bacilli, etc.

2. The purity of the cultivation; this can only be determined when gross morphological differences exist between the organisms present.

3. The presence or absence of spores, which show their typical refrangibility exceedingly well when examined by this method.

4. The presence or absence of mobility. In a hang-

ing-drop specimen some form of movement can practically always be observed, and its character must be carefully determined by noting the relative positions of adjacent micro-organisms.

(*a*) Brownian or molecular movement. Minute particles of solid matter (including bacteria), when suspended in a fluid, will always show a vibratory movement affecting the entire field, but never altering the relative positions of the bacteria. (Cocci exhibit this movement, but with the exception of the Micrococcus agilis, the cocci are non-motile.)

(*b*) Streaming movement. This is due to currents set up in the hanging drop as a result of jarring of the specimen or of evaporation, and although the relative position of the bacteria may vary, still the flowing movement of large numbers of organisms in some one direction will usually be sufficient to demonstrate the nature of this motion.

(*c*) Locomotive movement, or true motility, is determined by observing some one particular bacillus changing its position in the field independently of, and in a direction contrary to, other organisms present.

When the examination is completed and the specimen finished with, the "fresh specimen"—*i. e.*, the slide with the cover-slip attached—must be dropped into the lysol pot. In the hanging-drop specimen, however, the cover-slip only is infected, and this may be raised from the ring cell by means of forceps and dropped into the disinfectant.

Permanent Staining of the Hanging-drop Specimen.—Occasionally it is necessary to fix and stain a hanging-drop preparation. This may be done as follows:

1. Remove the cover-slip from the cell by the aid of the forceps.

2. If the drop is small, fix it by dropping it face downwards, whilst still wet, on to the surface of some Gulland's solution or corrosive sublimate solution (*vide*

page 76) in a watch-glass. If the drop is large, place it face upwards on the rubber mat, cover it with an inverted watch-glass, and allow it to dry. Then fix it in the alcohol and ether solution (*vide infra*).

3. Dip the cover-glass into a beaker containing hot water in order to remove some of the vaseline adhering to it.

4. Wash successively in alcohol, xylol, ether, and alcohol, to remove the last traces of grease.

5. Wash in water.

6. Stain, wash, dry, and mount as for an ordinary cover-slip film preparation (*vide* page 78).

2. Killed, Stained.—In this method three distinct processes are necessary:

1. "Preparing" and "fixing" the film.
2. Staining.
3. Mounting.

1*a*. *Preparing the Film.*—

1. Proceed as in making a hanging-drop preparation, steps 2 to 4.

2. Spread the drop of emulsion evenly over the cover-slip in the form of a square film to within 1 mm. of each edge of the cover-slip.

3. Allow it to dry completely in the air.

1*b*. *Fixing.*—Fix by passing the cover-slip, held in the fingers, three times through the flame of a Bunsen burner. In preparing films for staining, it is sometimes necessary (as in the case of those intended for micrometric observations) to fix by exposure to a uniform temperature of 115° C., for twenty minutes. This is best done in a carefully regulated hot-air oven. Fixation may also be effected by immersing in some fixative fluid, such as one of the following:

1. Absolute alcohol.
2. Absolute alcohol, Ether, } equal parts, for five to thirty minutes (*e. g.*, for blood or milk).

3. Osmic acid, 1 per cent. aqueous solution, for thirty seconds.

4. Corrosive sublimate, saturated aqueous solution, for five minutes.

5. Corrosive sublimate (Lang), for five minutes. This solution is prepared by dissolving:

Sodium chloride	0.75 gramme
Hydrarg. perchlor.	12.00 grammes
Acetic acid	5.00 "
In distilled water	100.00 c.c.

Filter.

6. Gulland's solution, for five minutes. This solution is prepared by mixing:

Absolute alcohol	25.0 c.c.
Ether	25.0 "
Corrosive sublimate, 20 per cent. alcoholic solution	0.4 "

Either of these methods of fixation coagulates the albuminous material and ensures perfect adhesion of the film to the cover-slip.

Wash the cover-slip thoroughly in running water and proceed with the staining.

If the film has been prepared from broth, liquefied gelatine, or pus or other morbid exudations, saturate the film after fixation with acetic acid 2 per cent. and allow it to act for two minutes.

Wash with alcohol, then let the alcohol remain on the cover-slip for two minutes. (This will "clear" the groundwork and give a much sharper and cleaner film than would otherwise be obtained.)

If the film has been prepared from blood or blood-stained fluid, treat with acetic acid 2 per cent. for two minutes after fixation. Wash with water, dry, and proceed with the staining. (This will remove the hæmoglobin and facilitate examination.)

2. *Staining.*—

1. Rest the cover-slip, film side uppermost, on the rubber mat.

2. By means of a drop-bottle, cover the film side of the cover-slip with the selected stain, allow it to act for a few minutes, then wash off the excess in running water.

The penetrating power of stains is increased by (*a*) physical means—*e. g.*, heating the stain; (*b*) chemical means—*e. g.*, by the addition of carbolic acid, 5 per cent. aqueous solution; caustic alkalies, 2 per cent. aqueous solution; water saturated with aniline oil; borax, 0.5 per cent. aqueous solution.

The most commonly used dyes for cover-slip film preparations are the aniline dyes.

(A) Basic:

(a) Methylene-blue.

(b) Gentian violet.

(c) Fuchsin.

These dyes are kept in saturated alcoholic (90 per cent.) solutions so that decomposition may be retarded.

Two or three drops of alcoholic solution of these dyes to, say, 4 c.c. water, usually makes a sufficiently strong staining fluid for cover-slip film preparations.

Carbolic methylene-blue (C.M.B.) and carbol fuchsin (C.F.) are prepared by covering the cover-slip with 5 per cent. solution of carbolic acid and adding a few drops of the saturated alcoholic solution of methylene-blue or fuchsin respectively to it. For aniline gentian violet (A.G.V.) the stain is added to a saturated solution of aniline oil in water.

(d) Thionin blue.

(e) Bismarck brown.

(B) Acid:

(a) Eosin, aqueous yellowish.

(b) Safranine.

These dyes are kept in 1 per cent. aqueous solution to which is added 5 per cent. of alcohol, as a preservative. They are generally used in this form.

A few nuclear stains (carmine, hæmatoxylin) are occasionally used more especially in "section" work.

2a. Decolourisation.—After overstaining, films may be decolourised by washing for a longer or shorter time in one of the following reagents arranged in ascending order of power

1. Water.
2. Chloroform.
3. Acetic acid, 1 per cent.
4. Alcohol.
5. Alcohol absolute, Acetic acid, 1 per cent., } equal parts.
6. Mineral acids: Hydrochloric, 1 per cent. aqueous solution. Hydrochloric, 1 per cent. alcoholic (90 per cent.) solution. Sulphuric, 25 per cent. aqueous solution. Nitric, 33 per cent. aqueous solution.

2b. Counterstaining.—Use colours which will contrast with the first stain; *e. g.*,

Vesuvin, Eosin, Fuchsin, } for films stained by methylene-blue or Gram's method.

Methylene-blue, Gentian violet, } for films stained by fuchsin.

3. *Mounting.*—

1. Wash the film carefully in running water.

2. Blot off the superfluous water with the filter paper, or dry more completely between two folds of blotting paper.

3. Complete the drying in the air, or by holding the cover-slip in the fingers at a safe distance above the flame of the Bunsen burner.

4. Place a drop of xylol balsam on the centre of a clean 3 by 1 glass slide and invert the cover-slip over

the balsam, and lower it carefully to avoid the inclusion of air bubbles.

NOTE.—Xylol is used in preference to chloroform to dissolve Canada balsam, as it does not decolourise the specimen.

Impression films (*Klatschpräparat*) are prepared from isolated colonies of bacteria in order that their characteristic formation may be examined by higher powers than can be brought to bear on the living cultivation. They are prepared from plate cultivations (*vide* page 181), in the following manner.

1. Remove a clean cover-slip from the alcohol pot with sterile forceps and burn off the spirit.

2. Open the plate and rest one edge of the cover-slip on the surface of the medium a little to one side of the selected colony. Lower it cautiously over the colony until horizontal. Avoid any lateral movement or the inclusion of bubbles of air.

3. Make gentle vertical pressure on the centre of the cover-slip with the points of the forceps to ensure perfect contact with the colony.

4. Steady one edge of the cover-slip with the forceps and pass the point of a mounted needle just under the opposite edge and raise the cover-slip carefully; the colony will be adherent to it. When nearly vertical, grasp the cover-slip with the forceps and remove it from the plate. Re-cover the plate.

5. Place the cover-slip, film uppermost, on the rubber mat, and cover it with an inverted watch-glass until dry.

6. Fix by immersing in one of the fixing fluids previously mentioned (*vide* page 75).

7. Clear with acetic acid and alcohol.

8. Stain and mount as an ordinary cover-slip film preparation, being careful to perform all washing operations with extreme gentleness.

Microscopical Examination of the Stained Specimen.—(The body tube of the microscope may be vertical or inclined to an angle.)

1. Secure the slide on the stage of the microscope by means of the spring clips.

2. Place a drop of cedarwood oil on the centre of the cover-slip.

3. Use the $\frac{1}{12}$-inch oil immersion lens of the microscope. Rack down the body tube till the front lens of the objective is in contact with the oil and nearly touching the cover-slip.

4. Rack up the condenser until it is in contact with the under surface of the slide.

5. Apply the eye to the ocular and arrange the plane mirror so as to obtain the greatest possible amount of light.

6. Rack up the body tube until the stained film comes into view.

7. Focus the condenser accurately on the film.

8. Focus the film accurately by means of the fine adjustment.

V. STAINING METHODS.

BACTERIA STAINS.

In the following pages are collected the various "stock" stains in everyday use in the bacteriological laboratory, together with a selection of the most convenient and generally useful staining methods for demonstrating particular structures or differentiating groups of bacteria. The stains employed should either be those prepared by Grübler, of Leipzig, or Merck, of Darmstadt. The methods printed in ordinary type are those which a long experience has shown to be the most reliable, and to give the best results

Methylene-blue.—

1. *Saturated Aqueous Solution.*

Weigh out

Methylene-blue . . . . . 1.5 grammes

Place in a stoppered bottle having a capacity of from 150 to 200 c.c. and add

Distilled water . . . . . 100.0 c.c.

Allow the water to remain in contact with the dye for two weeks, shaking the contents of the bottle vigourously for a few moments every day. Filter.

2. *Saturated Alcoholic Solution.*

Weigh out

Methylene-blue . . . . . 1.5 grammes

Place in a stoppered bottle of 150 c.c. capacity and add

Alcohol, 90 per cent. . . . . 100.0 c.c.

Allow the alcohol to remain in contact with the dye for two hours, shaking vigourously every few minutes. Filter.

3. *Carbolic Methylene-blue* (Kühne).

Weigh out

Methylene-blue	1.5 grammes
Carbolic acid	5.0 "

and dissolve in

Distilled water	100.0 c.c.

and add

Absolute alcohol	10.0 "

Filter.

4. *Alkaline Methylene-blue* (Löffler).

Measure out and mix

Methylene-blue, saturated alcoholic solution	30.0 c.c.
Caustic potash, 0.1 per cent. aqueous solution	100.0 "

Filter.

Fuchsin (Basic).—

5. *Saturated Aqueous Solution.*

Weigh out

Basic fuchsin	1.5 grammes

and proceed as in preparing the corresponding solution of methylene-blue (*q. v.*).

6. *Saturated Alcoholic Solution.*

Weigh out

Basic fuchsin	3.5 grammes

and proceed as in preparing the corresponding solution of methylene-blue.

7. *Carbolic Fuchsin* (Ziehl).

Weigh out

Basic fuchsin	1.0 gramme
Carbolic acid	5.0 grammes

dissolve in

Distilled water	100.0 c.c.

and add

Absolute alcohol	10.0 "

Filter.

Gentian Violet.—

8. *Saturated Aqueous Solution.*

Weigh out

Gentian violet	2.25 grammes

and proceed as in preparing the corresponding solution of methylene-blue.

9. *Saturated Alcoholic Solution.*

Weigh out

Gentian violet	5.0 grammes

and proceed as in preparing the corresponding solution of methylene-blue.

10. *Carbolic Gentian Violet* (Nicollé).

Measure out and mix

Gentian violet, saturated alcoholic solution . .	10.0 c.c.
Carbolic acid, 1 per cent. aqueous solution . .	100.0 "

Filter.

Thionine Blue (or Lauth's Violet).—

11. *Carbolic Thionine Blue* (Nicollé).

Weigh out

Thionine blue	1.0 gramme
Carbolic acid	2.5 grammes

and dissolve in

Distilled water	100.0 c.c.

Filter.

Before use dilute with equal quantity of distilled water and again filter.

CONTRAST STAINS.

Eosin.—There are several commercial varieties of eosin, which, from the bacteriological point of view, possess very different values. Grübler lists four varieties, of which two only are useful for bacteriological work:

Eosin, aqueous yellowish.

Eosin, aqueous bluish.

12. *Eosin Aqueous Solution* (Yellowish or Bluish Shade), 1 per cent.

Weigh out

Eosin, aqueous . . . . . 1.0 gramme

dissolve in

Distilled water . . . . . 100.0 c.c.

and add

Absolute alcohol . . . . . 5.0 "

Filter.

13. *Eosin Alcoholic Solution*, 0.5 per cent.

Weigh out

Eosin, alcoholic . . . . . 0.5 gramme

and dissolve in

Alcohol (70 per cent.) . . . . . 100.0 c.c.

Filter.

Vesuvin (or Bismarck Brown).—

14. *Saturated Aqueous Solution.*

Weigh out

Vesuvin . . . . . 0.5 gramme

and dissolve in

Distilled water . . . . . 100.0 c.c.

Filter.

Safranine.—

Weigh out

Safranine . . . . . 0.5 gramme

and dissolve in

Distilled water . . . . . 100.0 c.c.

Filter.

TISSUE STAINS.

Lithium Carmine (Orth).—

Weigh out

Carmine . . . . . 2.5 grammes

and dissolve in

Lithium carbonate, cold saturated solution . . . 100.0 c.c.

Filter.

Picrocarmine.—

Weigh out

Picrocarmine . . . . . 2.0 grammes

and dissolve in

Distilled water . . . . . 100.0 c.c.

Hæmatoxylin.—

1. Weigh out

Hæmatoxylin	2.0 grammes

and dissolve in

Absolute alcohol	100.0 c.c.

2. Weigh out

Ammonium alum	2.0 grammes

and dissolve in

Distilled water	100.0 c.c.

3. Mix 1 and 2, allow the mixture to stand forty-eight hours, then filter.

4. Add

Glycerine	85.0 c.c.
Acetic acid, glacial	10.0 "

5. Allow the stain to stand for one month; then filter ready for use.

Aniline Gentian Violet (For Weigert's Fibrin Stain).—

Weigh out

Gentian violet	1.0 gramme

and dissolve in

Absolute alcohol	15.0 c.c.
Distilled water	80.0 "

then add

Aniline oil	3.0 "

Shake well and filter before use.

Alum Carmine (Meyer).—

Weigh out

Alum	2.5 grammes
Carmine	1.0 gramme

and place in a glass beaker.

Measure out in a measuring cylinder,

Distilled water	100.0 c.c.

Place the beaker on a sand-bath, add the water in successive small quantities, and keep the mixture boiling for twenty minutes. Measure the solution and make up to 100 c.c. by the addition of distilled water. Filter.

METHODS OF DEMONSTRATING STRUCTURE OF BACTERIA.

To Demonstrate Capsules.

1. MacConkey.—

Stain.—

Weigh out

Dahlia	0.5 gramme
Methyl green (00 crystals)	1.5 grammes

rub up in a mortar with

Distilled water	100.0 c.c.

Add

Fuchsin, saturated alcoholic solution	10.0 "

and make up to 200 c.c. by the addition of

Distilled water	90.0 c.c.

Filter.

Allow the stain to stand for two weeks before use; keep in a dark place or in an amber glass bottle. Owing to the unstable character of the methyl green, this stain deteriorates after about six months.

METHOD.—

1. Prepare and fix film in the usual manner.
2. Flood the cover-slip with the stain and allow it to act for five to ten minutes.
3. Wash very thoroughly in water; if necessary, direct a powerful stream of water on the film from a wash-bottle.
4. Dry and mount.

2. Welch's Method.—

1. Prepare and fix film in the usual manner.
2. Flood the slide with acetic acid 2 per cent.; allow the acid to remain in contact with the film for two minutes. This swells up and fixes the capsule and enables it to take the stain.
3. Blow off the acetic acid by the aid of a pipette.
4. Immerse in aniline gentian violet, five to thirty seconds.
5. Wash in water.
6. Dry and mount.

3. Ribbert's Method.—

Stain.—

Measure out and mix

Acetic acid, glacial	12.5 c.c.
Alcohol, absolute	50.0 "
Distilled water	100.0 "

Warm to 36° C. (*e. g.*, in the "hot" incubator) and saturate with dahlia. Filter.

METHOD.—

1. Prepare and fix films in the usual manner.
2. Cover the film with the stain and allow it to act for one or two seconds only.
3. Wash thoroughly in water.
4. Dry and mount.

To Demonstrate Flagella.

1. Muir's Modified Pitfield.—This is the best method and gives the most reliable results, for not only is the percentage of successful preparations higher than with any other, but the bacilli and flagella retain their relative proportions.

(*a*) *Mordant.—*

Tannic acid, 10 per cent. aqueous solution	10 c.c.
Corrosive sublimate, saturated aqueous solution	5 "
Alum, saturated aqueous solution	5 "
Carbolic fuchsin (Ziehl)	5 "

Mix thoroughly.

A precipitate forms which must be allowed to settle for a few hours.

Decant off the clear fluid into tubes and centrifugalise thoroughly.

This solution keeps for about a couple of weeks, but must be re-centrifugalised each time, before use.

(*b*) *Stain.—*

Alum, saturated aqueous solution	25 c.c.
Gentian violet, saturated alcoholic solution	5 "

Filter.

This stain must be freshly prepared.

METHOD.—The cultivations employed should be smear agar cultures, twelve to eighteen hours old if

incubated at 37° C., twenty-four to thirty hours if incubated at 22° C.

1. Remove a very small quantity of the growth by means of the platinum spatula.

2. Emulsify it with a few cubic centimetres of distilled water in a watch-glass, by gently moving the spatula to and fro in the water. Do not rub up the growth on the side of the watch-glass.

3. Spread a thin film of the emulsion on a newly flamed cover-slip, using no force, but rather *leading* the drop over the cover-slip with the platinum loop.

4. Allow the film to dry in the air, properly protected from falling dust.

5. Fix by passing thrice through the Bunsen flame, holding the cover-slip whilst doing so by one corner between the finger and thumb.

6. Pour on the film as much of the mordant as the cover-glass will hold. Grasp the cover-slip with the forceps and hold it, high above the flame, until steam rises. Allow the steaming mordant to remain in contact with the film two minutes.

7. Wash well in water and dry carefully.

8. Pour on the film as much of the stain as the cover-glass will hold. Steam over the flame as before for two minutes.

9. Wash well in water.

10. Dry and mount.

2. "Pitfield" Original Method.—

(*a*) *Mordant.*—

Tannic acid	1 gramme
Water	10 c.c.

(*b*) *Stain.*—

Saturated aqueous solution of alum	10 c.c.
Saturated alcoholic solution of gentian violet	1 "
Distilled water	5 "

Mix equal parts of *a* and *b* before using.

1. Prepare and fix the film in the manner described above.

2. Boil the mixture and immerse the cover-slip in it, whilst still hot, for one minute.

3. Wash in water.

4. Examine in water; if satisfactory, dry and mount in Canada balsam.

3. MacCrorrie's Method.—

Mordant-Stain.—

Measure out and mix

Night blue, saturated alcoholic solution	10 c.c.
Potash alum, saturated aqueous solution	10 "
Tannin, 10 per cent. aqueous solution	10 "

NOTE.—The addition of gallic acid, 0.1 to 0.2 gramme, may improve the solution, but is not necessary.

METHOD.—

1. Prepare and fix the films as above.

2. Pour some of the mordant-stain on the film and warm gently, high above the flame, for two minutes (or place in the "hot" incubator for a like period).

3. Wash thoroughly in water.

4. Dry and mount.

4. Van Ermengem's Method.—This method, being merely a precipitation of a silver salt on the micro-organisms and not a true stain, is unsatisfactory; the relative proportions of bacteria and flagella are almost invariably destroyed.

(*a*) *Fixing Fluid.—*

Osmic acid, 2 per cent. aqueous solution	10 c.c.
Tannic acid, 20 per cent. aqueous solution . . .	20 "
Acetic acid, glacial	1 "

The fixing fluid should be prepared some days before use and filtered as required. In colour it should be distinctly violet.

(*b*) *Sensitising Solution.—*

Silver nitrate, 0.5 per cent. aqueous solution.

This solution must be kept in a dark blue glass bottle or in a dark cupboard.

Filter immediately before use.

(*c*) *Reducing Solution.—*

Weigh out

Gallic acid	5 grammes
Tannic acid	3 "
Potassium acetate, fused	10 "

and dissolve in

Distilled water	350 c.c.

Filter.

This solution will keep active for several days, but fresh solution must be used for each preparation.

METHOD.—

1. Prepare emulsion, make and fix films therefrom as described in the preceding method, steps 1 to 4.
2. Pour on the film as much of the fixing solution as the cover-glass will hold, heat carefully over the flame till steam rises, and allow the steaming fixing fluid to act for five minutes.
3. Wash well in water.
4. Wash in absolute alcohol.
5. Wash in distilled water.
6. Pour some of the sensitising solution on the film and allow it to act for from thirty seconds to one minute; blot off the excess of fluid with filter paper.
7. Without washing, transfer the film to a watch-glass containing the reducing solution and allow it to remain therein for from thirty seconds to one minute; blot off the excess of fluid with filter paper.
8. Without washing, again treat the film with the sensitising solution, this time until the film commences to turn black.
9. Wash in distilled water.
10. Dry and mount.

5. Löffler's Method.—

(*a*) *Mordant.*—

Tannic acid, 20 per cent. aqueous solution . . .	10 c.c.
Ferrous sulphate, saturated aqueous solution . .	5 "
Hæmatoxylin solution (prepared by boiling 1 gramme logwood with 8 c.c. distilled water, filtering and replacing the loss from evaporation)	3 "
Carbolic acid, 1 per cent. aqueous solution . . .	4 "

This solution must be freshly prepared.

Alternative Mordant.—

Tannic acid, 20 per cent. aqueous solution . . .	10 c.c.
Ferrous sulphate, saturated aqueous solution . .	5 "
Fuchsin, saturated alcoholic solution	1 "

(*b*) *Stain.*—

Weigh out

Methylene-blue	4 grammes
Or methylene-violet	
Or fuchsin	

and dissolve in

Aniline water, freshly saturated and filtered . .	100 c.c.

METHOD.—

1. Prepare and fix films as above.
2. Pour the mordant on to the film and warm cautiously over the flame till steam rises; keep the mordant gently steaming for one minute.
3. Wash well in distilled water till no more colour is discharged; if necessary, wash carefully with absolute alcohol.
4. Filter a few drops of the stain on to the film, warm as before, and allow the steaming stain to act for one minute.
5. Wash well in distilled water.
6. Dry and mount.

NOTE.—The flagella of some organisms can be demonstrated better by means of an alkaline stain or an acid stain —a point to be determined for each. According to requirements, therefore, Löffler recommends the addition of sodium hydrate, 1 per cent. aqueous solution, 1 c.c.; or an equal quantity of an exactly comparable solution of sulphuric acid.

To Stain Spores.

1. Single Stain.—

1. Prepare cover-slip film in the usual way.
2. In fixing, pass the cover-slip film fifteen or thirty times through the flame instead of only three. This destroys the resisting power of the spore membrane and allows the stain to reach the interior.
3. Stain in the usual way with methylene-blue or fuchsin.
4. Wash in water.
5. Dry and mount

2. Double Stain.—

1. Prepare and fix film in the usual way—*i. e.*, pass three times through flame to fix.
2. Cover the film with hot carbol-fuchsin and hold in the forceps above a small flame until the fluid begins to steam. Set the cover-slip down and allow it to cool. Repeat the process when the stain ceases to steam and continue to repeat until the stain has been in contact with the film for twenty minutes. (This stains both spores and bacteria.)
3. Wash in water.

4. Decolourise in alcohol, 2 parts; acetic acid, 1 per cent., 1 part. (This removes the stain from everything but the spores.)

5. Wash in water.

6. Mount the cover-slip in water and examine microscopically with the $\frac{1}{6}$-inch objective. (Spores should be red, and the rest of the film colourless or a very light pink.) If satisfactory, pass on to section 7; if unsatisfactory, repeat steps 2 to 5.

7. Counterstain in weak methylene-blue. (Now spores red, bacilli blue.)

8. Wash in water.

9. Dry and mount.

The spores of different bacilli differ greatly in their resistance to decolourising reagents; even the spores of the same species of organisms vary according to their age. Young spores are more easily decolourised than those more mature.

Sulphuric acid, 1 per cent. aqueous solution, and hydrochloric acid, 0.5 per cent. alcoholic (90 per cent.) solution, are useful decolourising reagents.

3. Muller's Method.—

1. Prepare and fix films in the usual manner.

2. Immerse in absolute alcohol for two minutes, then in chloroform for two minutes; wash in water. This dissolves out any fat or crystals that might otherwise retain the "spore" stain.

3. Immerse in chromic acid, 5 per cent. aqueous solution, for one minute; wash in water.

4. Pour Ziehl's carbolic fuchsin on the film, warm as in previous methods, and allow it to act for ten minutes.

5. Wash in water.

6. Decolourise in sulphuric acid, 5 per cent. aqueous solution, for five seconds.

7. Wash in water.

8. Counterstain with Kuhne's carbolic methylene-blue for one or two minutes.

9. Wash in water.

10. Dry and mount.

(Spores red, bacilli blue.)

4. Abbott's Method.—

1. Prepare and fix films in the usual manner.
2. Pour Löffler's alkaline methylene-blue on the film; warm cautiously over the flame till steam rises and allow the hot steam to act for one to five minutes.
3. Wash thoroughly in water.
4. Decolourise in nitric acid, 2 per cent. alcoholic (80 per cent.) solution.
5. Wash thoroughly in water.
6. Counterstain in eosin, 1 per cent. aqueous solution.
7. Wash.
8. Dry and mount.

(Spores blue, bacilli red.)

DIFFERENTIAL METHODS OF STAINING.

Gram's Method.—This method depends upon the fact that the protoplasm of some bacteria permits aniline gentian violet and Lugol's iodine solution, when applied consecutively, to enter into a chemical combination which results in the formation of a new blue-black pigment, only very sparingly soluble in alcohol. Such organisms are said to "stain by Gram."

1. Prepare a cover-slip film and fix in the usual way.
2. Stain in aniline gentian violet three to five minutes.

To prepare aniline water, pour 4 or 5 c.c. aniline oil into a stoppered bottle and add distilled water, 100 c.c. Shake vigourously and filter immediately before use. The excess of oil sinks to the bottom of the bottle and may be used again.

Filter as much aniline water on to the cover-slip as it will hold; then add the smallest quantity of alcoholic solution of gentian violet which suffices to saturate the aniline water and form a "bronze scum" upon its surface.

3. Wash in water.
4. Treat with Lugol's iodine solution until the film is black or dark brown.

To do this treat with iodine solution for a few seconds, wash in water, and examine the film over a piece of white filter paper. Note the colour. Repeat this

process until the film ceases to darken with the fresh application of iodine solution.

Lugol's solution is prepared by dissolving

Iodine	1 gramme
Iodine of potassium	3 grammes
In distilled water	300 c.c.

5. Wash in water.

6. Wash with alcohol until no more colour is discharged and the alcohol runs away clear and colourless.

7. Wash in water.

8. Counterstain very lightly with dilute eosin, dilute fuchsin, or vesuvin.

NOTE.—This section may be omitted when dealing with films prepared from pure cultivations.

9. Wash in water.

10. Dry and mount.

Gram-Weigert Method.—(Also extremely useful for sections.)

1–5. Proceed as for the corresponding sections of Gram's method (*quod vide*).

6. Dry in the air.

7. Wash in aniline oil, 1 part, xylol, 2 parts, until no more colour is discharged.

8. Wash in xylol.

9. Mount in xylol balsam.

(Then fibrin and hyaline tissue are stained deep blue, whilst bacteria which "stain Gram" appear of a deep blue-violet colour.)

Modified Gram-Weigert Method.—(Employed to demonstrate trichophyta in hair.)

1. Soak the hairs in ether for ten minutes to remove the fat.

2. Stain ten to sixty minutes in a tar-like solution of aniline gentian violet (prepared by adding 15 drops of the alcoholic solution of gentian violet to 3 drops of aniline water).

3. Dry the hairs between pieces of blotting paper.

4. Treat with perfectly fresh iodine solution.

5. Again dry between blotting paper.

6. Treat with aniline oil. (If necessary, add a drop or two of nitric acid to the oil.)

7. Again treat with aniline oil.

8. Treat with aniline oil and xylol, equal parts.

9. Clear with xylol.

10. Mount in xylol balsam.

To Differentiate the Tubercle Bacillus and Other Acid-fast Bacilli (Ziehl-Neelsen's Method).—

1. Smear a thin, even film of the specimen on the cover-slip by means of the platinum loop. (In the case of sputum, if it is a very watery specimen, allow the film to dry, then spread a second and even a third layer over the first.)

2. Fix by passing three times through the flame.

3. Stain in hot carbol-fuchsin (as in staining for spores) for five to ten minutes. (This stains everything on the film.)

4. Decolourise by dipping in sulphuric acid, 25 per cent. (This removes stain from everything but acid-fast bacilli; *e. g.*, tubercle, leprosy, and smegma bacilli.)

5. Wash in water.

6. Wash in alcohol till no more colour is discharged. (This usually, but not invariably, removes the stain from acid-fast bacilli other than tubercle; *e. g.*, smegma bacillus.)

7. Wash in water.

8. Counterstain in weak methylene-blue. (Stains non-acid-fast bacilli, leucocytes, epithelial cells, etc.)

9. Wash in water, dry, and mount.

To Differentiate the Diphtheria Bacillus (Neisser's Method).—

Stain I.—

Weigh out

Methylene-blue . . . . . 1 gramme

and dissolve in

95 per cent. alcohol	20 c.c.
Distilled water	950 "

then add

Acetic acid (glacial)	50 "

Filter.

Stain II.—

Weigh out

Vesuvin	2 grammes

and dissolve in

Distilled water, boiling	1000 c.c.

Cool and filter.

METHOD.—

1. Prepare and fix films in the usual way.
2. Pour stain I on the film and allow it to act for thirty seconds.
3. Wash thoroughly in water.
4. Pour stain II on to the film and allow it to act for thirty seconds.
5. Wash thoroughly in water.
6. Dry and mount.

NOTE.—The cultivation from which the films are prepared must be upon blood-serum which has been incubated at 37° C. for from nine to eighteen hours.

The bacilli are stained a light brown by the vesuvin, which contrasts well with the two or three black spots, situated at the poles and occasionally one in the centre representing protoplasmic aggregations (? meta-chromatic granules) stained by the acid methylene-blue.

To Demonstrate the (?) Syphilis Bacillus (Lustgarten's Method).—

1. Prepare and fix the film in the usual manner.
2. Stain in aniline gentian violet or aniline fuchsin, twenty-four hours.
3. Wash in water.
4. Place in 1.5 per cent. solution of permanganate of

potash (a brown precipitate of oxide of manganese forms), three to four seconds.

5. Wash in watery solution of pure SO_2. If not decolourised, repeat sections 4 and 5 until satisfactory.

6. Wash in water, dry, and mount.

Or—

1. Prepare and fix the film in the usual manner.

2. Stain in hot carbol-fuchsin.

3. Wash in water to which has been added 2 or 3 drops of chloride of lime.

4. Decolourise in concentrated solution of chloride of iron.

5. Wash thoroughly in water.

6. Dry and mount.

Syphilis bacilli remain red.

NOTE.—These methods are equally applicable to tissue sections.

VI. METHODS OF DEMONSTRATING BACTERIA IN TISSUES.

For bacteriological purposes, sections of tissues are most conveniently prepared by either the **freezing method** or the **paraffin method.**

The latter is decidedly preferable, but as it is of greater importance to demonstrate the bacteria, if such are present, than to preserve the tissue elements unaltered, the "frozen" sections are often of value.

Whichever method is selected, it is necessary to take small pieces of the tissue for sectioning,—0.5 c.c. cubes when possible,—not exceeding half a centimetre in thickness. Post-mortem material should be secured as soon after the death of the animal as possible.

The tissue is prepared for cutting by—

(*a*) Fixation; that is, by causing the death of the cellular elements in such a manner that they retain their characteristic shape and form.

The fixing fluids in general use are: Absolute alcohol; corrosive sublimate, saturated aqueous solution; corrosive sublimate, Lang's solution (*vide* page 76); formaldehyde, 1 per cent. aqueous solution. (Of these, Lang's corrosive sublimate solution is decidedly the best all-round "fixative.")

(*b*) Hardening; that is, by rendering the tissue of sufficient consistency to admit of thin slices or "sections" being cut from it. This is effected by passing the tissue successively through alcohols of gradually increasing strength: 30 per cent. alcohol, 50 per cent. alcohol, 75 per cent. alcohol, 90 per cent. alcohol, rectified alcohol, absolute alcohol.

In both these processes a large excess of fluid should always be used.

FREEZING METHOD.

1. **Fixation.** Place the pieces of tissue in a wide-mouthed glass bottle and fill with absolute alcohol. Allow the tissues to remain therein for twenty-four hours.

2. **Hardening.** Remove the alcohol (no longer absolute, as it has taken up water from the tissues) from the bottle and replace it with fresh absolute alcohol. Allow the tissues to remain therein for twenty-four hours.

NOTE.—If not needed for cutting immediately, the hardened tissues can be stored in 50 per cent. alcohol.

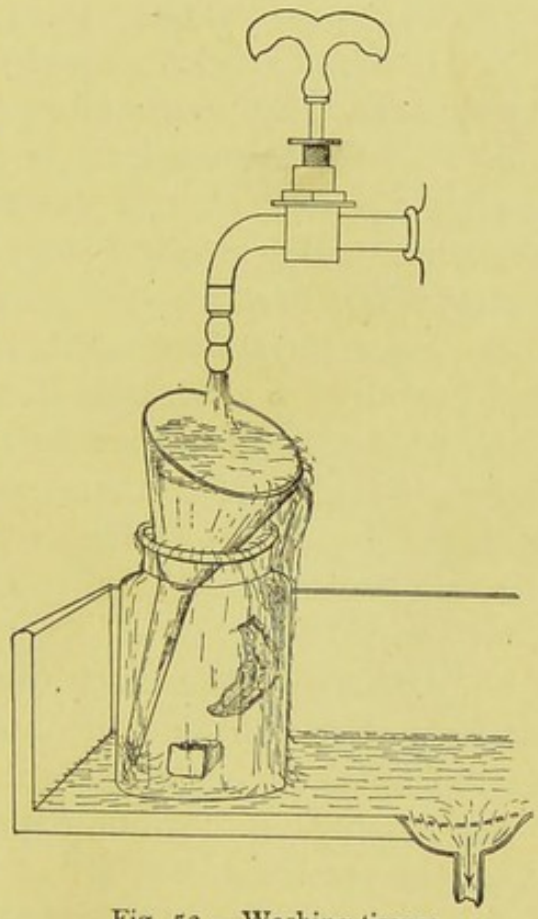


Fig. 53.—Washing tissues.

3. Remove the alcohol from the tissues by soaking in water from one to two hours. Remove the stopper from the bottle; rest a glass funnel in the open mouth and place under a tap of running water. The water, of course, overflows, but the tissues remain in the bottle.

4. Impregnate the tissues with mucilage for twelve to twenty-four hours, according to size. Transfer the pieces of tissue to a bottle containing sterilised gum mixture.

Formula.—

Gum arabic	5 grammes
Saccharose	1 gramme
Boric acid	1 "
Water	100 c.c.

5. Place the tissue on the plate of a freezing microtome (Cathcart's is perhaps the best form), cover and surround with fresh gum mixture; freeze, and cut sections.

6. Float the sections off the knife into a glass dish containing tepid water and allow them to remain therein for about an hour to dissolve out the gum.

(If not required at once, store in 50 per cent. alcohol.)

7. Transfer to a glass capsule containing the selected staining fluid, by means of a section lifter.

8. Transfer the sections in turn to a capsule containing absolute alcohol (to dehydrate) and to one containing xylol or oil of cloves (to clear).

Alternative Method.—

8*a*. Place the stained section in a dish of clean water and introduce a glass slide obliquely beneath the section; with a mounted needle draw the section on to the slide and hold it there; gently remove the slide from the water, taking care that any folds in the section are floated out before the slide is finally removed from the water.

8*b*. Drain away as much water as possible from the section. Drop absolute alcohol on to the section from a drop bottle, to dehydrate it.

8*c*. Double a piece of blotting paper and gently press it on the section to dry it.

8*d*. Drop on xylol to clear the section.

9. Place a large drop of xylol balsam on the section and carefully lower a cover-glass on to the balsam.

PARAFFIN METHOD.

1. **Fixation.** Place the pieces of tissue, resting on cotton-wool, in a wide-mouthed glass bottle. Pour on a sufficient quantity of the corrosive sublimate fixing fluid; allow the tissue to remain therein for twelve to twenty-four hours according to size.

2. Pour off the fixing fluid and wash thoroughly in running water for twenty minutes to half an hour to remove the excess of corrosive sublimate.

3. **Hardening.** Place the tissues in each of the following strengths of alcohol in turn for from twelve to twenty-four hours: 50 per cent., 75 per cent., 90 per cent., absolute.

4. Dehydrate by transferring to fresh absolute alcohol.

5. Clear by immersing in equal parts of absolute alcohol and xylol for six to eighteen hours; then in pure xylol for from fifteen minutes to six hours. The tissue should not be allowed to remain in the xylol longer than is necessary. When "cleared," the tissue becomes more or less transparent.

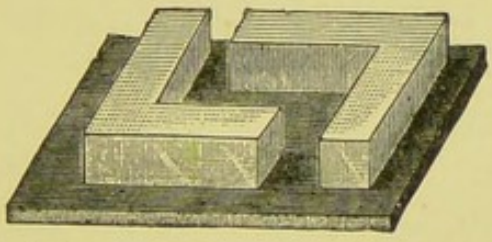


Fig. 54.—L-shaped brass moulds.

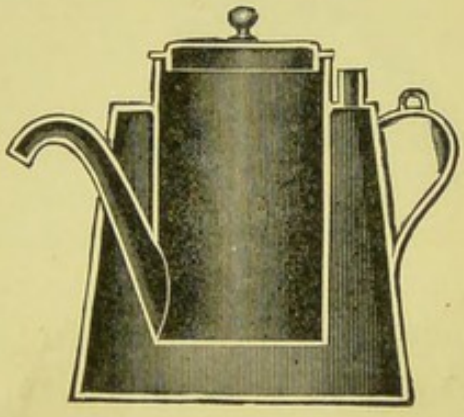


Fig. 55.—Paraffin kettle.

6. Transfer the tissues to a vessel containing melted paraffin. Place this vessel in a paraffin water-bath regulated for 2° C. above the melting-point of the paraffin used and allow the tissues to soak for some six to twelve hours to ensure complete impregnation. The paraffin used should have a melting-point of not more than 58° C. For all ordinary purposes 54° C. will be found quite high enough.

7. Imbed in fresh paraffin in a metal (or paper) mould.

(*a*) Arrange a pair of L-shaped pieces of metal on a plate of glass to form a rectangular trough (Fig. 54).

(*b*) Pour fresh melted paraffin into the mould from a special vessel (Fig. 55).

(*c*) Lift the piece of tissue from the paraffin bath and arrange it in the mould.

(*d*) Blow gently on the surface of the paraffin in the mould, and as soon as a film of solid paraffin has formed, carefully lift the glass plate on which the mould is set and lower plate and mould together into a basin of cold water.

(*e*) When the block is cold, break off the metal L's; trim off the excess of paraffin from around the tissue with a knife, taking care to retain the rectangular shape, and store the block in a pill-box.

When several pieces of tissue have to be imbedded at one time, shapes of copper, 10 cm., 5 cm., and 2.5 cm. square respectively, and 0.75 cm. deep (Fig. 56) will be found extremely useful. These placed upon plates of glass replace the pair of L's in the above process.

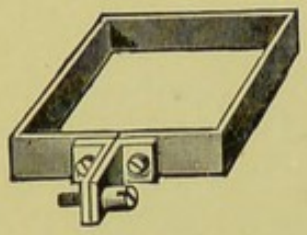


Fig. 56.—Paraffin mould.

8. Cement the block on the carrier of a "paraffin" microtome (the Minot, the Jung, or the Cambridge Rocker) with a little melted paraffin. Greater security is obtained if the paraffin around the base of the block is melted by means of a hot metal or glass rod.

9. Cut sections—thin, and if possible in ribbands.

Mounting Paraffin Sections.—

1. Place a large drop of 30 per cent. alcohol on the centre of a slide (or cover-slip) and float the section on to the surface of the drop, from a section lifter.

2. Hold the slide in the fingers of one hand and warm cautiously over the flame of a Bunsen burner, touching the under surface of the glass from time to time on the back of the other hand. As soon as the slide

feels distinctly warm to the skin, the paraffin section will flatten out and all wrinkles disappear.

(The slide with the section floating on it may be rested on the top of the paraffin bath for two or three minutes, instead of warming over the flame as here described.)

3. Cautiously tilt up the slide and blot off the excess of spirit with blotting paper, leaving the section attached to the centre of the slide.

4. Place the slide in a wire rack (Fig. 57), section downwards, in the "hot" incubator for twelve to twenty-four hours. At the end of this time the section is firmly adherent to the glass, and is treated during the subsequent steps as a "fixed" cover-glass film preparation.

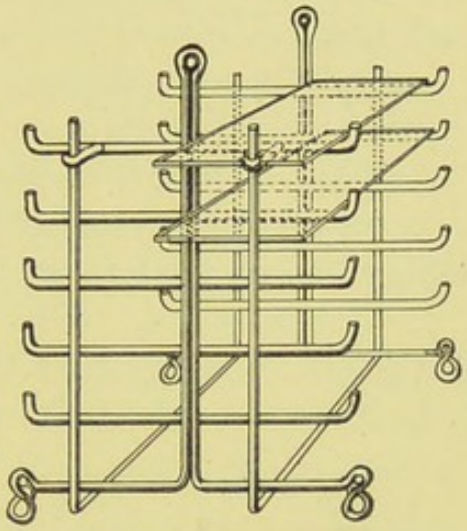


Fig. 57.—Section rack.

NOTE.—If large, thick sections have to be manipulated, or if time is of importance, it is often advisable to add a trace of Mayer's albumin to the alcohol before floating out the section. If this substance is employed, a sojourn of twenty minutes to half an hour in the "hot" incubator will be found ample to ensure firm adhesion of the section to the slide. Mayer's albumin is prepared as follows:

Weigh out

Salicylate of soda . . . . . 1 gramme

and dissolve in

Glycerine . . . . . 50 c.c.

Add

White of egg . . . . . 50 "

Mix thoroughly by means of an egg whisk.
Filter into a clean bottle.

Staining Paraffin Sections.—

1. Warm paraffin section over the Bunsen flame to

soften (*but not to melt*) the paraffin, then dissolve out the wax with xylol.

2. Remove xylol by flushing the section with alcohol.

3. If the tissue was originally "fixed" in a corrosive sublimate solution, the section must now be treated with Lugol's iodine solution for five minutes.

4. Stain deeply, if using a single stain, as the subsequent processes decolourise.

5. Wash in water, decolourise if necessary.

6. Flood with several changes of absolute alcohol to dehydrate the section.

7. Clear in xylol. (Oil of cloves is not usually employed, as it decolourises the section.)

8. Mount in xylol balsam.

SPECIAL STAINING METHODS FOR SECTIONS.

Double-staining Carmine and Gram-Weigert.—

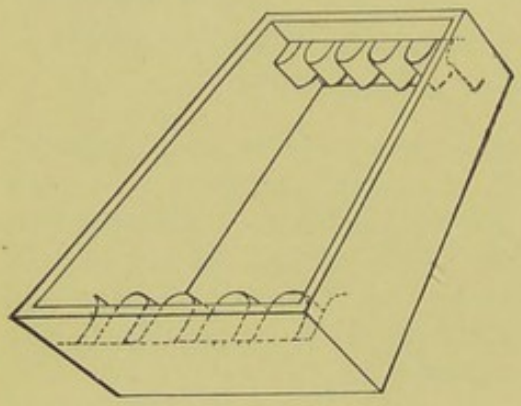


Fig. 58.—Staining pot.

1. Prepare the section for staining as above, sections 1 to 3.

2. Stain in lithium carmine (Orth's) or picrocarmine for ten to thirty minutes in a porcelain staining pot (Fig. 58).

3. Wash in picric acid solution until yellow. At this stage cell nuclei are red, protoplasm is yellow, and bacteria are colourless.

Picric acid solution is prepared by mixing

Picric acid, saturated aqueous solution	40 c.c.
Hydrochloric acid	1 "
Alcohol (90 per cent.)	160 "

4. Wash in water.

5. Wash in alcohol.

6. Stain in aniline gentian violet.

7. Wash in iodine solution till dark brown or black.

8. Wash in water.

9. Dip in absolute alcohol for a second.

10. Decolourise with aniline oil till no more colour is discharged.

11. Wash with aniline oil, 2 parts, xylol, 1 part.

12. Clear with xylol.

13. Mount in xylol balsam.

Alternative Gram-Weigert Method for Sections.—

1. Fix paraffin section on slide and prepare for staining in the usual manner.

2. Stain in alum carmine for about fifteen minutes.

3. Wash thoroughly in water.

4. Filter aniline gentian violet solution on to the section on the slide and allow to stain about twenty-five minutes.

5. Wash thoroughly in water.

6. Treat with Lugol's iodine until section ceases to become any blacker.

7. Wash thoroughly in water.

8. Treat with a mixture of equal parts of aniline oil and xylol until no more colour comes away.

9. Wash thoroughly with xylol.

10. Decolourise and dehydrate rapidly with absolute alcohol until there remains only a very faint bluish tint.

11. Clear with xylol.

12. Mount in xylol balsam.

To Demonstrate Capsules.—

1. *MacConkey's Method.*—Stain precisely as for cover-slip films.

2. *Friedländer's Method.*—

Stain.—

Gentian violet, saturated alcoholic solution . . .	50 c.c.
Acetic acid, glacial	10 "
Distilled water	100 "

METHOD.—

1. Prepare the sections for staining, *secundum artem.*
2. Stain sections in the warm (*e. g.*, in the hot incubator) for twenty-four hours.
3. Wash with water.
4. Decolourise lightly with acetic acid, 1 per cent.
5. Dehydrate rapidly with absolute alcohol.
6. Clear with xylol.
7. Mount in xylol balsam.

To Demonstrate Acid-fast Bacilli.—

1. Prepare the sections for staining in the usual way.
2. Stain with hæmatin solution ten to twenty seconds, to obtain a pure nuclear stain; then wash in water.
3. Stain with carbolic fuchsin twenty to thirty minutes at 47° C.; then wash in water.
4. Treat with aniline hydrochlorate, 2 per cent. aqueous solution, for two to five seconds.
5. Decolourise in 75 per cent. alcohol till section appears free from stain—fifteen to thirty minutes.
6. Dehydrate with absolute alcohol.
7. Clear very rapidly with xylol.
8. Mount in xylol balsam.

VII. CLASSIFICATION OF FUNGI.

FUNGI are divided into:

1. **Hymenomycetes** (including the mushrooms, etc.).
2. **Hyphomycetes** (moulds).
3. **Blastomycetes** (yeasts and torulæ).
4. **Schizomycetes** (bacteria).

NOTE.—Formerly myxomycetes were included in the fungi; they are now recognised as belonging to the animal kingdom, and are termed "mycetozoa."

MORPHOLOGY OF THE HYPHOMYCETES.

At the commencement of his studies, the attention of the student is directed to the various non-pathogenic moulds and yeasts, not only that he may gain the necessary technique whilst handling cultivations of harmless organisms, but also because these very species are amongst the commonest of those that may accidentally contaminate his future preparations.

The hyphomycetes are composed of a mycelium of short jointed rods or "hyphæ" springing from an axis or germinal tube which develops from the spore. Hyphæ are—

(*a*) Nutritive or submerged.

(*b*) Reproductive or aerial.

The protoplasm of these cells contains granules, pigment, oil globules, and sometimes crystals of calcium oxalate.

Reproduction.—Apical spore formation—asexual; zoospores—sexual.

Mucorinæ.—*Mucor* (Fig. 59).—Note the branching filaments—"mycelium" (*a*), "hyphæ" (*b*).

Note the asexual reproduction.

1. A filament grows upwards. At its apex a septum forms, then a globular swelling appears—"sporagium" (*d*). This possesses a definite membrane.

2. From the septum grows a club-shaped mass of protoplasm—"columella" (*c*).

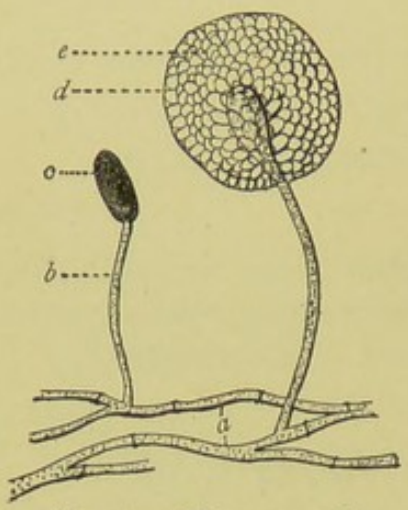


Fig. 59.—Mucor mucedo.

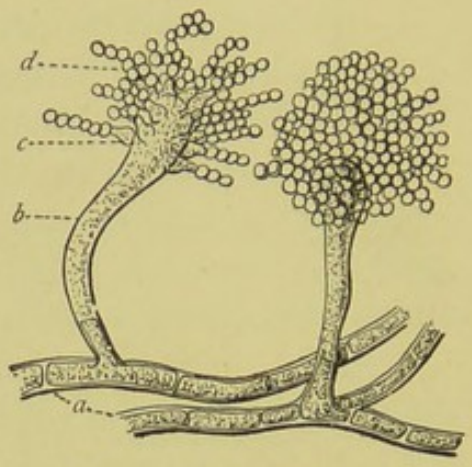


Fig. 60.—Aspergillus.

3. The rest of the contained protoplasm breaks up into "swarm spores" (*e*).

Finally the membrane ruptures and spores escape.

Perisporaceæ.—*Aspergillus* (Fig. 60).—Note the branching filaments—"mycelium" (*a*).

Note the asexual reproduction.

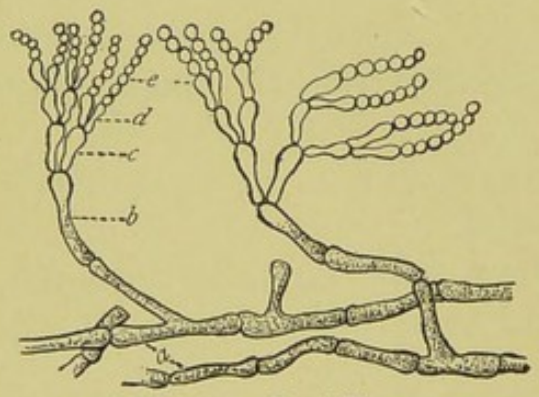


Fig. 61.—Penicillium.

1. A filament (*b*) grows upwards, its termination becomes clubbed; on the clubbed extremity flask-shaped cells appear—"sterigmata" (*c*).

2. At free end of each sterigma is formed an oval body—a spore or "gonidium" (*d*), which, when ripe, is thrown off from the sterigma.

Penicillium (Fig. 61).—Note the branching filaments —"mycelium" (*a*) (frequently containing globules).

Note the asexual reproduction.

1. A filament grows upwards—"goniodophore" (*b*)—and its apex divides up into several branches—"basidia"(*c*).

2. At the apex of each branch a flask-shaped cell, "sterigma" (*d*), appears.

3. At the apex of each sterigma appears a row of oval cells—"spores" or "conidia" (*e*). These, when ripe, are cast off from the sterigmata.

Ascomycetæ.—*Oïdium* (Fig. 62).—(This family is perhaps as nearly related to the blastomycetes as it is to the hyphomycetes.)

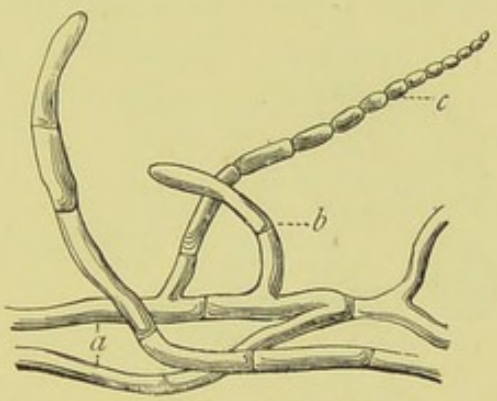


Fig. 62.—Oïdium.

Note the branching filaments—"pseudomycelium" (*a*). Here and there filaments are broken up at their ends into oval or rod-shaped segments, "oïdia," and behave as spores.

Note the asexual reproduction. From the pseudomycelium arise true hyphæ (*b*), each of which in turn ends in a chain of spores (*c*).

MORPHOLOGY OF THE BLASTOMYCETES.

The blastomycetes are composed of spherical or oval cells (8 to 9.5 μ in diameter), which, when rapidly multiplying by budding, may form a spurious mycelium. A thin cell-wall encloses the granular protoplasm, in which vacuoles and sometimes a nucleus may be noted. This latter is best seen when stained with osmic acid or hæmatoxylin.

During their growth and multiplication the blasto-

mycetes split up solutions containing sugar into alcohol and CO_2.

Reproduction.—Budding, ascospores—asexual.

(Torulæ, whilst resembling yeasts in almost every other respect, never form spores.)

Saccharomyces (Fig. 63).—Note the round or oval cells of granular protoplasm (*a*) containing solid particles and vacuoles (*c*), and surrounded by a definite envelope.

Note the asexual *reproduction.*

1. "Gemmation"—that is, the budding out of daughter cells (*b*) from various parts of the gradually enlarging mother cell. These are eventually cast off

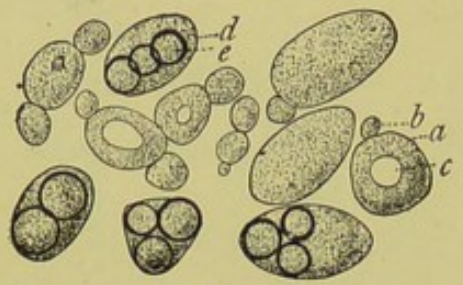


Fig. 63.—Saccharomyces with ascospores.

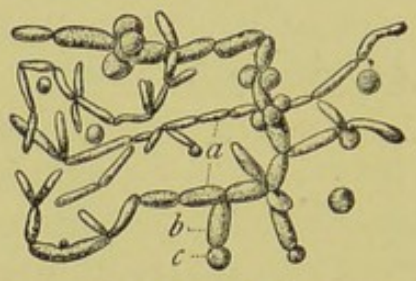


Fig. 64.—Torula.

and in turn become mother cells and form fresh groups of buds.

2. Spore formation—"ascospores" (*e*). These are formed at definite temperatures and within well-defined periods; *e. g.*, Saccharomyces cerevisiæ, thirty hours at 25° to 37° C., or ten days at 12° C.

Torulæ (Fig. 64).—Note the points in morphology, etc., as above.

Note the absence of ascospore formation.

VIII. SCHIZOMYCETES.

Classification and Morphology.—Bacteria are often classified, in general terms, according to their life functions, into—

Saprogenic, or putrefactive bacteria;
Zymogenic, or fermentative bacteria;
Pathogenic, or disease-producing bacteria;

or according to their metabolic products into—

Chromogenic, or pigment-producing bacteria;
Photogenic, or light-producing bacteria;
Aerogenic, or gas-producing bacteria;

and so on.

Such broad groupings as these have, however, but little practical value when applied to the systematic study of the fission fungi.

On the other hand, no really scientific classification of the schizomycetes has yet been drawn up, and the varying morphological appearances of the members of the family are still utilised as a basis for classification, as under—

1. *Micrococci, or Cocci* (Fig. 65).—Rounded or oval cells, subdivided according to the arrangement of the individuals after fission, into—

Diplococci and Streptococci, where division takes place in one plane only, and the individuals remain attached (*a*) in pairs or (*b*) in chains.

Tetrads, Merismopedia, or Pediococci, where division takes place alternately in two planes at right angles to each other, and the individuals remain attached in flat tablets of four, or its multiples.

Sarcinæ, where division takes place in three planes successively, and the individuals remain attached in cubical packets of eight and its multiples.

Staphylococci, where division takes place in three planes, but with no definite sequence; consequently the individuals remain attached in pairs, short chains, plates of four, cubical packets of eight, and irregular masses containing numerous cocci.

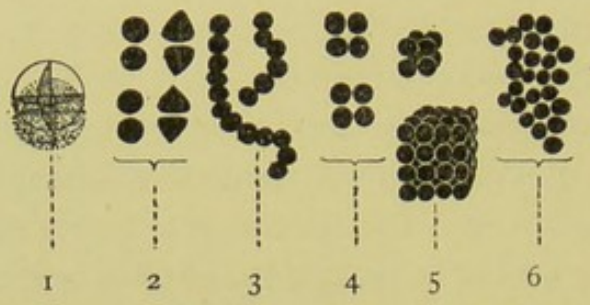


Fig. 65.—Types of bacteria—cocci: 1, Diagram of sphere indicating planes of fission; 2, diplococci; 3, streptococci; 4, tetrads; 5, sarcinæ; 6, staphylococci.

2. *Bacilli* (Fig. 66, 1 to 3).—Rod-shaped cells. A bacillus, however short, can usually be distinguished from a coccus in that two sides are parallel. Some bacilli after fission retain a characteristic arrangement and may be spoken of as diplobacilli or streptobacilli.

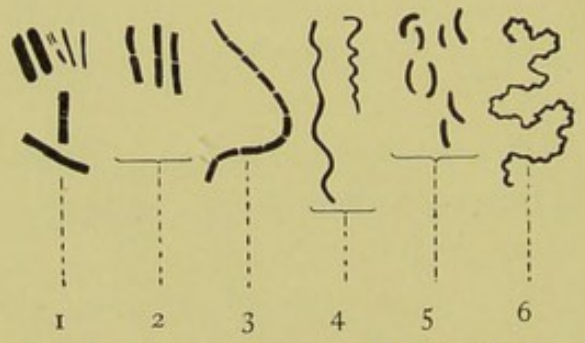


Fig. 66.—Types of bacteria—bacilli, etc.: 1, Bacilli; 2, diplobacilli; 3, streptobacilli; 4, spirilla; 5, vibrios; 6, spirochætæ.

(Leptothrix is a term that in the past has been loosely used to signify a long thread, but is now restricted to such forms as belong to the leptothriciæ (*vide* page 113).

3. *Spirilla* (Fig. 66, 4 to 6).—Curved and twisted filaments. Classified, according to shape, into—

Spirillum.
Vibrio (comma).
Spirochæta.

Higher forms of bacteria are also met with, which possess the following characteristics: They are attached, unbranched, filamentous forms, showing—

(a) Differentiation between base and apex;
(b) Growth apparently apical;
(c) Exaggerated pleomorphism;
(d) "Pseudo-branching" from apposition of cells;

and are classified into—

1. Beggiotoa. } Free swimming forms, which contain
2. Thiothrix. } sulphur granules.
3. Crenothrix. } These forms do not contain sulphur
4. Cladothrix. } granules.
5. Leptothrix. }

6. Streptothrix. A group which exhibits true branching, and contains some pathogenic species.

The morphology of the same bacterium may vary greatly under different conditions of growth as to—

1. The composition, reaction, etc., of the *nutrient medium.*
2. *The atmosphere* in which it is cultivated.
3. *The temperature* at which it is incubated.
4. Exposure to or protection from *light.*

For example, under one set of conditions the examination of a pure cultivation of a bacillus may show a short oval rod as the predominant form, whilst another culture of the same bacillus, but grown under different conditions, may consist almost entirely of long filaments or threads, a condition known as "pleomorphism."

ANATOMY.

1. *Capsule* (Fig. 67, *b*).—A gelatinous envelope (probably akin to mucin in composition) surrounding each individual organism, and preventing absolute con-

tact between any two. In some species the capsule is well marked, but it cannot be demonstrated in all. In very well marked cases of gelatinisation of the cell wall, the individual cells are cemented together in a coherent mass, to which the term "zoogloœa" is applied. In some species colouring matter or ferric oxide is stored in the capsule.

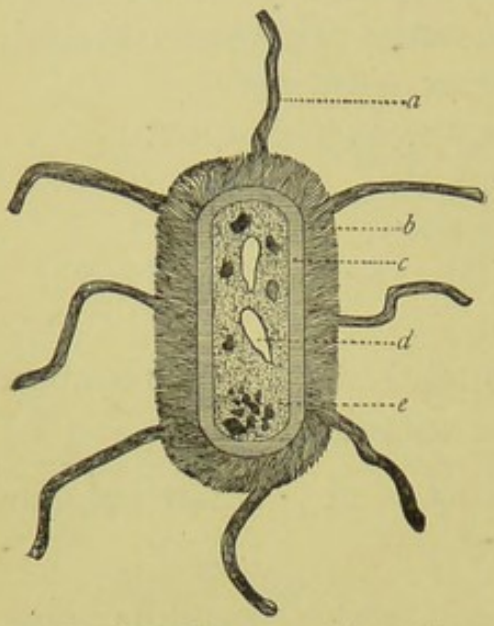


Fig. 67.—Structure of bacteria.

2. *Cell Wall* (Fig. 67, *c*). —A protective differentiation of the outer layer of the cell protoplasm; difficult to demonstrate, but treatment with iodine or salt solution sometimes causes shrinkage of the cell contents—"plasmolysis"—and so renders the cell wall apparent (*e. g.*, B. megatherium) in the manner shown in figure 68. Stained bacilli, when examined with polarising microscope, often show a doubly refractile cell wall (*e. g.*, B. tuberculosis and B. anthracis).

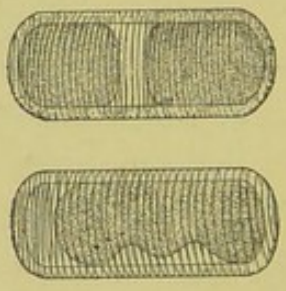


Fig. 68.—Plasmolysis.

In some of the higher bacteria the cell wall exhibits this differentiation to a marked degree and forms a hard sheath within which the cell protoplasm is freely movable; and during the process of reproduction the cell protoplasm may be extruded, leaving the empty tube unaltered in shape.

3. *Cell Contents.*—Protoplasm (mycoprotein) contains a high percentage of nitrogen, but is said to differ from proteid in that it is not precipitated by C_2H_6O. It is usually homogeneous in appearance—sometimes

granular—and may contain oil globules or sap vacuoles (Fig. 67, *d*), chromatin granules, and even sulphur granules. Sap vacuoles must be distinguished from spores, on the one hand, and the vacuolated appearance due to plasmolysis, on the other.

The cell contents may sometimes be differentiated into a parietal layer, and a central body (*e. g.*, beggiotoa) when stained by hæmatoxylin.

4. *Nucleus.*—This structure has not been conclusively proved to exist, but in some bacteria denser masses of protoplasm situated at the poles and exhibiting a more marked affinity than the rest of the cell protoplasm for aniline dyes have been observed. These are termed polar granules or *Polkörner* (Fig. 67, *e*). Occasionally these aggregations of protoplasm alter the colour of the dye they take up. They are then known as metachromatic bodies or *Ernst'schen Körner.*

5. *Flagella* (Organs of Locomotion, Fig. 67, *a*).—These are gelatinous elongations of the cell protoplasm (or more probably of the capsule), occurring either at one pole, at both poles, or scattered around the entire periphery. Flagella are not pseudopodia. The possession of flagella was at one time suggested as a basis for a system of classification, when the following types of ciliation were differentiated:

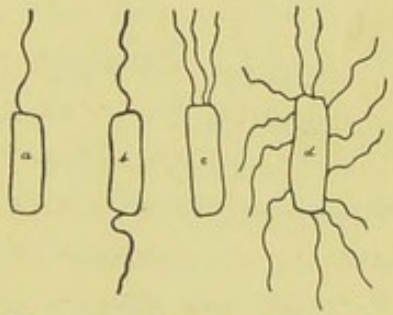


Fig. 69.—Types of ciliation.

1. Polar: (*a*) *Monotrichous* (a single flagellum situated at one pole; *e. g.*, B. pyocyaneus).

(*b*) *Amphitrichous* (a single flagellum at each pole; *e. g.*, Spirillum volutans).

(*c*) *Lophotrichous* (a tuft or bunch of flagella situated at each pole; *e. g.*, B. cyanogenus).

2. Diffuse: *Peritrichous* (flagella scattered around the entire periphery; *e. g.*, B. typhosus).

PHYSIOLOGY.

Reproduction.—*Active Stage.*—Vegetative, *i. e.*, by the division of cells, or "fission."

1. The cell becomes elongated and the protoplasm aggregated at opposite poles.

2. A circular constriction of the organism takes place midway between these aggregations, and a septum is formed in the interior of the cell at right angles to its length.

3. The division deepens, the septum divides into two lamellæ, and finally two cells are formed.

4. The daughter cells may remain united by the gelatinous envelope for a variable time. Eventually they separate and themselves subdivide.

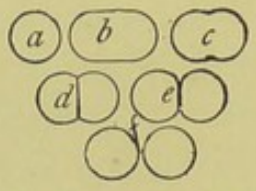


Fig. 70.—Fission of cocci.

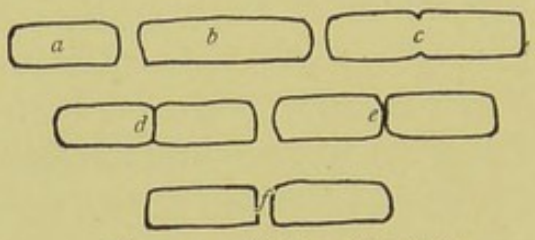


Fig. 71.—Fission of bacteria.

Cultures on artificial media, after growing in the same medium for some time,—*i. e.*, when the pabulum is exhausted,—show "involution forms" (Fig. 72), well exemplified in cultures of B. pestis on agar two days old, B. diphtheriæ on potato four to six days old.

They are of two classes, viz.:

(*a*) Involution forms characterised by alterations of shape. (Not necessarily dead.)

(*b*) Involution forms characterised by loss of staining power. (Always dead.)

Resting Stage.—Spore Formation.—Conditions influencing spore formation: In an old culture nothing may be left but spores. It used to be supposed that spores were *always* formed when

(*a*) The supply of nutrient was exhausted.

(*b*) The medium became toxic from the accumulation of metabolic products.

(*c*) The environment became unfavourable· *e. g.*, change of temperature.

So that the species might not become extinct. This is not altogether correct; *e. g.*, the temperature at which spores are best formed is constant for each bacterium, but varies with different species; again, aerobes require oxygen for sporulation, but anaerobes will not spore in its presence.

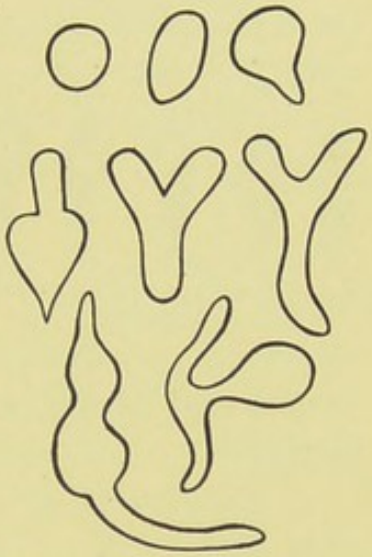


Fig. 72.—Involution forms.

(A) Arthrogenous: Noted only in the micrococci. One complete element resulting from ordinary fission becomes differentiated for the purpose, enlarges, and develops a dense cell wall. One or more of the cells in a series may undergo this alteration.

This process is probably not real spore formation, but merely relative increase of resistance. These so-called arthrospores have never been observed to "germinate," nor is their resistance very marked, as they fail to initiate new cultures, after having been exposed to a temperature of 80° C. for ten minutes.

(B) Endogenous: The cell protoplasm becomes differentiated and condensed into a spherical or oval mass (very rarely cylindrical). After further contraction the outer layers of the mass become still more highly differentiated and form a distinct spore membrane, and the spore itself is now highly refractile. It has been suggested, and apparently on good grounds, that the spore membrane consists of two layers, the exosporium and the endosporium. Each cell forms one spore only, usually in the middle, occasionally at

one end (four exceptions, however, are recorded; *e. g.*, B. inflatus). The shape of the parent cell may be unaltered, as in the anthrax bacillus, or altered, as in the tetanus bacillus, and these points serve as the basis for a classification of spore-bearing bacilli, as follows:

Fig. 73.—Types of spore-bearing bacilli.

(A) Cell body of the parent bacillus unaltered in shape (Fig. 73, *a*).

(B) Cell body of the parent bacillus altered in shape.

1. *Clostridium* (Fig. 73, *b*): Rod swollen at the centre and attenuated at the poles; spindle shape; *e. g.*, B. butyricus.

2. *Cuneate* (Fig. 73, *c*): Rods swollen slightly at one pole and more or less pointed at the other; wedge-shaped.

3. *Clavate* (Fig. 73, *d*): Rods swollen at one pole and cylindrical (unaltered) at the other; keyhole-shaped; *e. g.*, B. chauvei.

4. *Capitate* (Fig. 73, *e*): Rods with a spherical enlargement at one pole; drumstick-shaped; *e. g.*, B. tetani.

The endospores remain within the parent cell for a variable time (in one case it is stated that germination of the spore occurs within the interior of the parent cell—"endo-germination"), but are eventually set free, as a result of the swelling up and solution of the cell membrane of the parent bacillus in the surrounding liquid, or of the rupture of that membrane. They then present the following characteristics:

1. Well-formed, dense cell membranes, which renders them extremely difficult to stain, but when once stained equally difficult to decolourise.

2. High refractility, which distinguished them from vacuoles.

3. Higher resistance than the parent organism to

such lethal agents as heat, desiccation, starvation, time, etc., this resistance being due to

(*a*) Low water contents of plasma
(*b*) Low heat-conducting power
(*c*) Low permeability

} of the spore membrane.

This resistance varies somewhat with the particular species,—*e. g.*, some spores may resist boiling for a few minutes,—but practically all are killed if the boiling is continued for ten minutes.

Germination.—When transplanted to suitable media and placed under favourable conditions, the spores germinate, usually within twenty-four to thirty-six hours, and successively undergo the following changes:

1. Swell up slowly and enlarge, through the absorption of water.

2. Lose their refrangibility.

3. At this stage one of three processes (but the particular process is always constant for the same species) may be observed:

(*a*) The spore grows out into the new bacillus without discarding the spore membrane (which in this case now becomes the cell membrane); *e. g.*, B. leptosporus.

(*b*) It loses its spore membrane by solution; *e. g.*, B. anthracis.

(*c*) It loses its spore membrane by rupture.

In this process the rupture may be either polar, and taking place at one pole only (*e. g.*, B. butyricus) or occurring at both poles (*e. g.*, B. sessile), or equatorial; *e. g.*, B. subtilis.

In those cases where the spore membrane is discarded the cell membrane of the new bacillus may either be formed from—

(*a*) The inner layer of the spore membrane, which has undergone a preliminary splitting into parietal and visceral layers; *e. g.*, B. butyricus.

(*b*) The outer layers of the cell protoplasm, which

become differentiated for that purpose; *e. g.*, B. megatherium.

The new bacillus now increases in size, elongates,

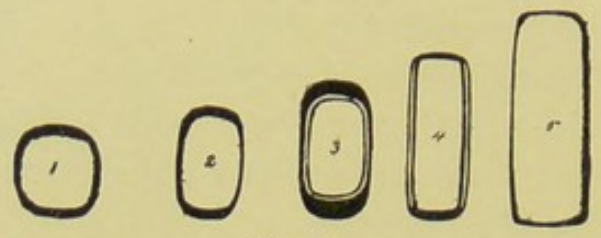


Fig. 74.

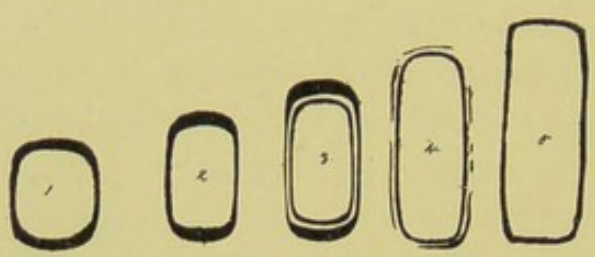


Fig. 75.

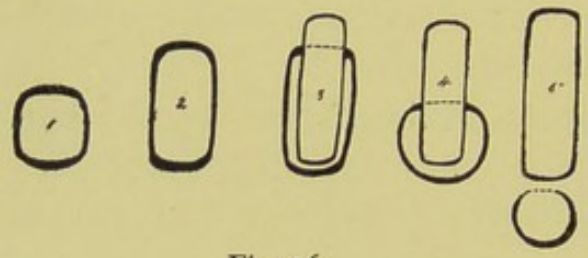


Fig. 76.

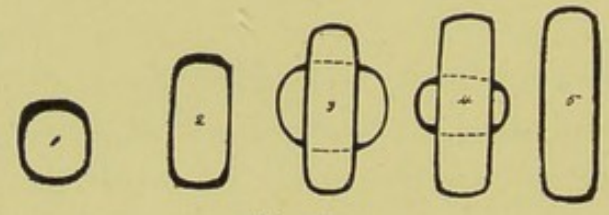


Fig. 77.

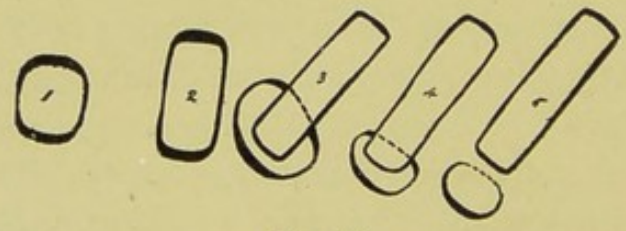


Fig. 78.

Figs. 74, 75, 76, 77, 78.—Types of germination: Fig. 74, simple; Fig. 75, solution; Fig. 76, polar; Fig. 77, bipolar; Fig. 78, equatorial.

and takes on a vegetative growth,—*i. e.*, undergoes fission,—the bacilli resulting from which may in their turn give rise to spores.

Food Stuffs.—1. *Organic Foods.*—

(*a*) The pure parasites (*e. g.*, B. lepræ) will not live outside the living body.

(*b*) Both saprophytic and facultative parasitic bacteria agree in requiring non-concentrated food.

(*c*) The facultative parasites need highly organised foods; *e. g.*, proteids or other sources of nitrogen and carbon, and salts.

(*d*) The saprophytic bacteria are more easily cultivated; *e. g.*,

1. Some bacteria will grow in almost pure distilled water.

2. Some bacteria will grow in pure solutions of the carbohydrates.

2. *Water* is absolutely essential to the *growth* of bacteria.

Food of a definite reaction is needed for the growth of bacteria. As a general rule growth is most active in media which react slightly acid to phenolphthalein —that is, neutral or faintly alkaline to litmus. Mould growth, on the other hand, is most vigourous in media that are strongly acid to phenolphthalein.

Environment.—The influence of physical agents upon bacterial life and growth is strongly marked.

1. *Atmosphere.*—The presence of *oxygen* is necessary for the growth of some bacteria, and death follows when the supply is cut off. Such organisms are termed *obligate aerobes.*

Some bacteria appear to thrive equally well whether supplied with or deprived of oxygen. These are termed *facultative anaerobes.*

A third class will only live and multiply when the access of free oxygen is completely excluded. These are termed *obligate anaerobes.*

2. *Temperature.*—Practically no bacterial growth occurs below 5° C., and very little above 40° C. 30° C. to 37° C. is the most favorable for the large majority of micro-organisms.

The maximum and minimum temperatures at which growth takes place, as well as the optimum, are fairly constant for each bacterium.

Bacteria have been classified, according to their optimum temperature, into—

	Min.	Opt.	Max.
1. Psychrophilic bacteria (chiefly water organisms)	0° C	15° C.	30° C.
2. Mesophilic bacteria (includes pathogenic bacteria)	15° C.	27° C.	45° C.
3. Thermophilic bacteria	45° C.	55° C.	70° C.

The thermal death-point of an organism is another biological constant; and is that temperature which causes the death of the vegetative forms when the exposure is continued for a period of ten minutes.

3. *Light.*—Many organisms are indifferent to the presence of light. On the other hand, light frequently impedes growth, and alters to a greater or lesser extent the biochemical characters of the organisms—*e. g.*, chromogenicity or power of liquefaction. Pathogenic bacteria undergo a progressive loss of virulence when cultivated in the presence of light.

4. *Movements.*—Movements, if slight and simply of a flowing character, do not appear to injuriously affect the growth of bacteria; but violent agitation, such as shaking, absolutely kills them.

A condition of perfect rest would seem to be that most conducive to bacterial growth.

The Metabolic Products of Bacteria.—*Pigment Production.*—Many micro-organisms produce one or more vivid pigments—yellow, orange, red, violet, fluorescent, etc.—during the course of their life and growth. The colouring matter usually exists as an intercellular

excrementitious substance. Occasionally, however, it appears to be stored actually within the bodies of the bacteria. The chromogenic bacteria are therefore classified, in accordance with the final destination of the colouring matter they elaborate, into—

Chromoparous Bacteria: in which the pigment is diffused out upon and into the surrounding medium.

Chromophorous Bacteria: in which the pigment is stored in the cell protoplasm of the organism.

Parachromophorous Bacteria: in which the pigment is stored in the cell wall of the organism.

Different species of chromogenic bacteria differ in their requirements as to environment, for the production of their characteristic pigments; *e. g.*, some need oxygen, light, or high temperature; others again favor the converse of these conditions.

Light Production.—Some bacteria, and usually those originally derived from water, whether fresh or salt, exhibit marked phosphorescence when cultivated under suitable conditions. These are classed as "photogenic."

Enzyme Production.—Many bacteria produce soluble ferments or enzymes during the course of their growth, as evidenced by the liquefaction of gelatine, the clotting of milk, etc. These ferments may belong to either of the following well-recognised classes: proteolytic, diastatic, invertin, rennet.

Toxin Production.—A large number, especially of the pathogenic bacteria, elaborate or secrete poisonous substances concerning which but little exact knowledge is available, although many would appear to be enzymic in their action.

These toxins are usually differentiated into—

Extracellular (or Soluble) Toxins: those which are diffused into, and held in solution by, the surrounding medium.

Intracellular (or Insoluble) Toxins: those which are

so closely bound up with the cell protoplasm of the bacteria elaborating them that up to the present time no means has been devised for their separation or extraction.

End-products of Metabolism.—Under this heading are included—

Organic Acids (*e. g.*, lactic, butyric, etc.).

Alkalies (*e. g.*, ammonia).

Aromatic Compounds (*e. g.*, indol, phenol).

Reducing Substances (*e. g.*, those reducing nitrates to nitrites).

Gases (*e. g.*, sulphuretted hydrogen, carbon dioxide, etc.).

And while the discussion of their formation, etc., is beyond the scope of a laboratory handbook, the methods in use for their detection and separation come into the ordinary routine work and will therefore be described (*vide* page 221 *et seq.*).

IX. NUTRIENT MEDIA.

In order that the life and growth of bacteria may be accurately observed in the laboratory, it is necessary—

1. To *isolate* individual members of the different varieties of micro-organisms.

2. To *cultivate* isolated organisms apart from other associated or contaminating bacteria—*i. e.*, in *pure culture*.

For the successful achievement of these objects it is necessary to provide nutriment in a form suited to the needs of the particular bacterium or bacteria under observation, and in a general way it may be said that the nutrient materials should approximate as closely as possible, in composition and character, to the natural soil of the organism.

The general requirements of bacteria as to their food-supply have already been indicated (page 121) and many combinations of proteids and of carbohydrates have been devised, from time to time, on these lines. These, together with various vegetable tissues, physiological or pathological fluid secretions, etc., are collectively spoken of as *nutrient media* or *culture media*.

The greater number of these media are primarily *fluid*, but, on account of the rapidity with which bacterial growth diffuses itself through a liquid, it is impossible to study therein the characteristics of individual organisms. Many such media are, therefore, subsequently rendered solid by the addition of substances like gelatine or agar, in varying proportions, the proportions of such added material being generally mentioned when referring to the media; *e. g.*, 10 per cent. gelatine, 2 per cent. agar. Gelatine is employed

for the solidification of those media it is intended to use in the cultivation of bacteria at the room temperature or in the "cold" incubator. In the percentages usually employed, gelatine media become fluid at 25° C.; higher percentages remain solid at somewhat higher temperatures, but the difficulty of filtering strong solutions of gelatine militates against their general use.

Agar media, on the other hand (even in 2 per cent. solutions), only become liquid when exposed to 90° C. for a considerable period, and again solidify at 40° C.

When it becomes necessary to render these media fluid, heat is applied, upon the withdrawal of which they again assume their solid condition. Such media should be referred to as *liquefiable media;* in point of fact, however, they are usually grouped together with the solid media.

NOTE.—It must here be stated that the designation 10 per cent. gelatine or 2 per cent. agar refers only to the quantity of those substances actually added in the process of manufacture, and *not* to the percentage of gelatine or agar, as the case may be, present in the finished medium; the explanation being that the commercial products employed contain a large proportion of insoluble material which is separated off by filtration during the preparation of the liquefiable media.

Other media, again,—*e. g.*, potato, coagulated blood-serum, etc.,—cannot be again liquefied by physical means, and these are spoken of as *solid* media.

The following pages detail the method of preparing the various nutrient media, those in general use being printed in bolder type than those occasionally required for more highly specialised work. It must be premised that scrupulous cleanliness is to be observed with regard to all apparatus, vessels, funnels, etc., employed in the preparation of media.

MEAT EXTRACT.

A watery solution of the extractives, etc., of lean meat (usually beef) forms the basis of several nutrient media. This solution is termed "meat extract," and is prepared as follows:

1. Measure 1000 c.c. of distilled water into a large flask (or glass beaker, or enamelled iron pot) and add 500 grammes (roughly, 1¼ pounds) of fresh lean meat, —*e. g.*, beefsteak or bullock's heart,—finely minced in a mincing machine.

2. Heat the mixture gently in a water-bath, taking care that the temperature of the contents of the flask does not exceed 40° C. for the first twenty minutes. (This dissolves out the soluble proteids, extractives, salts, etc.)

3. Now raise the temperature of the mixture to the boiling-point, and maintain at this temperature for ten minutes. (This precipitates some of the albumins, the hæmoglobin, etc., from the solution.)

4. Strain the mixture through sterile butter muslin or a perforated porcelain funnel, then filter the liquid through Swedish filter paper into a sterile "normal" litre flask, and when cold make up to 1000 c.c. by the addition of distilled water—to replace the loss from evaporation.

5. If not needed at once, sterilise the meat extract in bulk in the steam steriliser for twenty minutes on each of three consecutive days.

Calf, sheep, or chicken flesh is occasionally substituted for the beef; or the meat extract may be prepared from animal viscera, such as brain, spleen, liver, or kidneys.

NOTE.—As an alternative method, 3 grammes of Wyeth's beef juice, invalid bovril, or Liebig's extract of meat may be dissolved in 1000 c.c. distilled water, and heated and filtered as above.

Media prepared from such meat extracts are, however, eminently unsatisfactory when used for the cultivation of the more highly parasitic bacteria.

Reaction of Meat Extract.—Meat extract thus prepared is acid in its reaction, owing to the presence of acid phosphates of potassium and sodium, weak acids of the glycolic series, and organic compounds in which the acid character predominates. Owing to the nature of the substances from which it derives its reaction, the total acidity of meat extract can only be estimated accurately when the solution is at the boiling-point. Moreover, it has been observed that prolonged boiling (such as is involved in the preparation of nutrient media) causes it to undergo hydrolytic changes which increase its acidity, and the meat extract only becomes stable in this respect after it has been maintained at the boiling-point for forty-five minutes.

Although meat extract always reacts acid to phenolphthalein, it occasionally reacts neutral or even alkaline to litmus; and again, meat extract that has been rendered exactly neutral to litmus still reacts acid to phenolphthalein. This peculiar behaviour depends upon two factors:

1. Litmus is insensitive to many weak organic acids the presence of which is readily indicated by phenolphthalein.

2. Dibasic sodium phosphate which is formed during the process of neutralisation is a salt which reacts alkaline to litmus, but neutral to phenolphthalein.

In order, therefore, to obtain an accurate estimation of the reaction of any given sample of meat extract, it is essential that—

1. The meat extract be previously exposed to a temperature of 100° C. for forty-five minutes.

2. The estimation be performed at the boiling-point.

3. Phenolphthalein be used as the indicator.

The estimation is carried out by means of titration

experiments against standard solutions of caustic soda, in the following manner:

Method of Estimating the Reaction.—

Apparatus Required:

1. 25 c.c. burette graduated in tenths of a centimetre.
2. 1 c.c. pipette graduated in hundredths, and provided with rubber tube, pinch-cock, and delivery nozzle.
3. 25 c.c. measure (cylinder or pipette, calibrated for 98° C.—*not* 15° C.).
4. Several 60 c.c. conical beakers or Erlenmeyer flasks.
5. White porcelain evaporating basin, filled with boiling water and arranged over a gas flame as a water-bath.
6. Bohemian glass flask, fitted as a wash-bottle, and filled with distilled water, which is kept boiling on a tripod stand.

Solutions Required:

1. 10N NaOH, accurately standardised.
2. $\frac{n}{1}$ NaOH, accurately standardised.
3. $\frac{n}{10}$ NaOH, accurately standardised.
4. 0.5 per cent. solution of phenolphthalein in 50 per cent. alcohol.

METHOD.—Arrange the apparatus as indicated in figure 79.

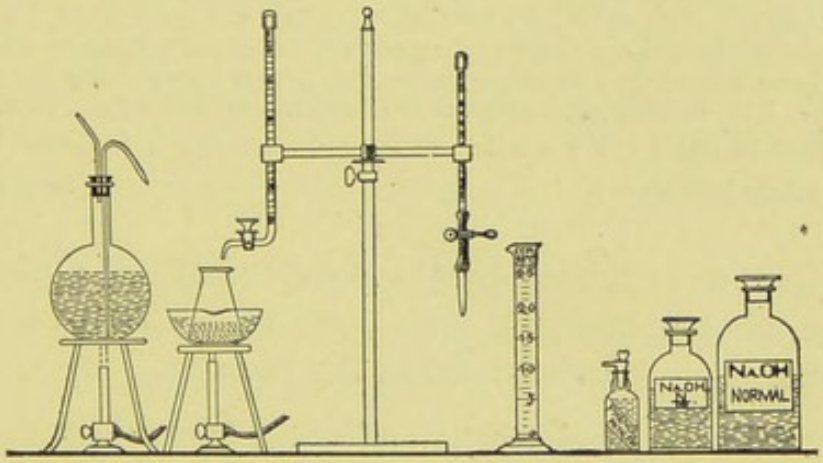


Fig. 79.—Arrangement of apparatus for titrating.

(A) 1. Fill the burette with $\frac{n}{10}$ NaOH.

2. Fill the pipette with $\frac{n}{1}$ NaOH.

3. Measure 25 c.c. of the meat extract (previously heated in the steamer at 100° C. for forty-five minutes)

into one of the beakers by means of the measure; rinse out the measure with a very small quantity of boiling distilled water from the wash-bottle, and then add this rinse water to the meat extract already in the beaker.

4. Run in about 0.5 c.c. of the phenolphthalein solution and immerse the beaker in the water-bath, and raise to the boil.

5. To the medium in the beaker run in $\frac{n}{10}$ NaOH cautiously from the burette until the end-point is

a *b* *c*

Fig. 80.—*a*, Sample of filtered meat extract or nutrient gelatine to which phenolphthalein has been added. The medium is acid, as evidenced by the unaltered colour of the sample. *b*, The same neutralised by the addition of $\frac{n}{10}$ NaOH. The production of this faint rose-pink colour indicates that the "end-point," or neutral point to phenolphthalein, has been reached. If such a sample is cooled down to say 30° or 20° C., the colour will be found to become more distinct and decidedly deeper and brighter, resembling that shown in *c*. *c*, Also if, after the end-point is reached, a further 0.5 c.c. or 1.0 c.c. $\frac{n}{10}$ NaOH be added to the sample, the marked alkalinity is evidenced by the deep colour here shown.

reached, as indicated by the development of a pinkish tinge, shown in figure 80. Note the amount of decinormal soda solution used in the process.

NOTE.—Just before the end-point is reached, a very slight opalescence may be noted in the fluid, due to the precipitation of dibasic phosphates. After the true end-point is reached, the further addition of about 0.5 c.c. of the decinormal soda solution will produce a deep magenta colour (Fig. 80, *c*), which is the so-called "end-point" of the American Committee of Bacteriologists.

(B) Perform a "control" titration (occasionally two controls may be necessary), as follows:

1. Measure 25 c.c. of the meat extract into one of the beakers, wash out the measure with boiling water, and add the phenolphthalein as in the first estimation.

2. Run in $\frac{n}{1}$ NaOH from the pipette, just short of the amount required to neutralise the 25 c.c. of medium. (For example, if in the first estimation 5 c.c. of $\frac{n}{10}$ NaOH were required to render 25 c.c. of medium neutral to phenolphthalein, only add 0.48 c.c. of $\frac{n}{1}$ NaOH.) Immerse the beaker in the water-bath.

3. Complete the titration by the aid of the $\frac{n}{10}$ NaOH.

4. Note the amount of $\frac{n}{10}$ NaOH solution required to complete the titration, and add it to the equivalent of the $\frac{n}{1}$ NaOH solution previously run in. Take the total as the correct estimation.

Method of Expressing the Reaction.—

The reaction or *titre* of meat extract, medium, or any solution estimated in the foregoing manner, is most conveniently expressed by indicating the number of cubic centimetres of normal alkali (or normal acid) that would be required to render *one litre* of the solution exactly neutral to phenolphthalein.

The sign + (plus) is prefixed to this number if the solution reacts acid, and the sign — (minus) if it reacts alkaline.

For example, "meat extract + 10," indicates a sample of meat extract which reacts acid to phenolphthalein, and would require the addition of 10 c.c. of normal NaOH per litre, to neutralise it.

NOTE.—Such a solution would probably react alkaline to litmus.

Conversely, if as the result of our titration experiments we find that 25 c.c. of meat extract require the addition of 5 c.c. $\frac{n}{10}$ NaOH to neutralise, then 1000 c.c.

of meat extract will require the addition of 200 c.c. $\frac{n}{10}$ NaOH = 20 c.c. $\frac{n}{1}$ NaOH.

And this last figure, 20, preceded by the sign +, to signify that it is acid, indicates the reaction of the meat extract.

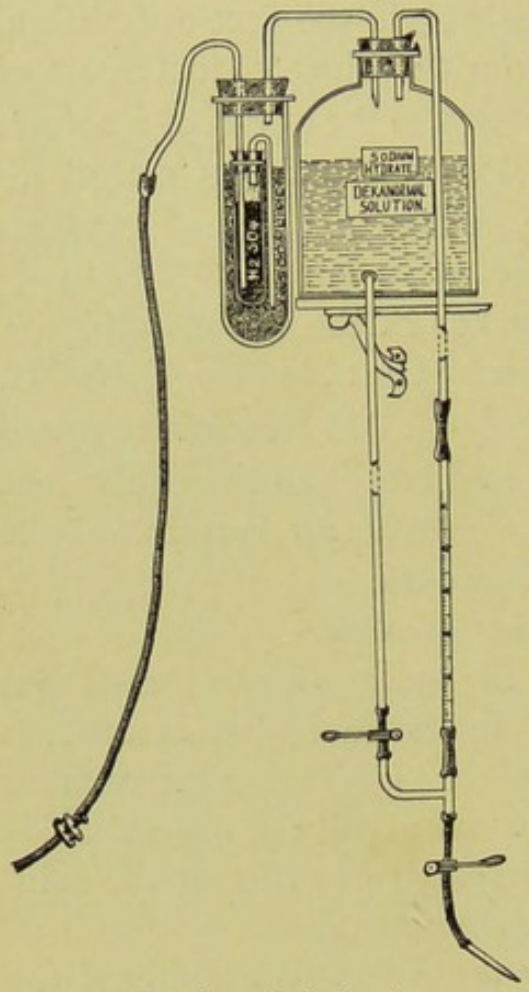


Fig. 81.—Soda bottle.

NOTE.—The standard soda solutions should be prepared by accurate measuring operations, controlled by titrations, from a stock solution of 10N NaOH, which should be very carefully standardised. This stock solution must be kept in an aspirator bottle to which air can only gain access after it has been dried and rendered free from CO_2. This may be done by first leading it over H_2SO_4 and soda lime, or soda lime alone, by some such arrangement as is shown in figure 81, which also shows a constant burette arrangement for the delivery of small measured quantities of the dekanormal soda solution.

STANDARDISATION OF MEDIA.

Differences in the reaction of the medium in which it is grown will provoke not only differences in the rate of growth of any given bacterium, but also well-marked differences in its cultural and morphological characters; and nearly every organism will be found to affect a definite "optimum reaction"—a point to be carefully determined for each. Fortunately, however, the "opti-

mum" usually approximates fairly closely to +10; and as experiment has shown that this reaction is the most generally useful for laboratory work, it is the one which may be adopted as the standard for all nutrient media derived from meat extract.

Briefly, the method of standardising a litre of media to +10 consists in subtracting 10 from the initial *titre* of the medium mass; the remainder indicates the number of cubic centimetres of normal soda solution that must be added to the medium, per litre, to render the reaction +10.

Standardising Nutrient Bouillon.—For example, 1000 c.c. bouillon are prepared; on titration it is found

1. 25 c.c. require the addition of 5.50 c.c. $\frac{n}{10}$ NaOH to neutralise.

2. 25 c.c. require the addition of 5.70 c.c. $\frac{n}{10}$ NaOH to neutralise.

3. 25 c.c. require the addition of 5.60 c.c. $\frac{n}{10}$ NaOH to neutralise.

2 and 3 are controls. Averaging these two controls, 25 c.c. require the addition of 5.65 c.c. $\frac{n}{10}$ NaOH to neutralise, and therefore 1000 c.c. require the addition of 226 c.c. $\frac{n}{10}$ NaOH, or 22.60 $\frac{n}{1}$ NaOH.

Initial *titre* of the bouillon = +22.6, and as such requires the addition of (22.6 c.c. — 10 c.c.) = 12.6 c.c. of $\frac{n}{1}$ NaOH per litre to leave its finished reaction +10.

But the three titrations, each on 25 c.c. of medium, have reduced the original bulk of the bouillon to (1000 — 75 c.c.) = 925 c.c. The amount of $\frac{n}{1}$ NaOH required to render the reaction of this quantity of medium +10 may be deduced thus:

$$1000 \text{ c.c.} : 925 \text{ c.c.} :: 12.6 \text{ c.c.} : x.$$

Then x = 11.65 c.c. $\frac{n}{1}$ NaOH.

Whenever possible, however, the required reaction is produced by the addition of dekanormal soda solution, on account of the minute increase it causes in the

bulk, and the consequent insignificant disturbance of the percentage composition of the medium. By means of a pipette graduated to 0.01 c.c. it is possible to deliver very small quantities; but if the calculated amount runs into thousandth parts of a cubic centimetre, these are replaced by corresponding quantities of normal soda.

In the above example it is necessary to add 11.65 c.c. normal NaOH or its equivalent, 1.165 c.c. dekanormal NaOH. The first being too bulky a quantity, and the second inconveniently small for exact measurement, the total weight of soda is obtained by substituting 1.16 c.c. dekanormal soda solution, and 0.05 c.c. of normal soda solution.

Standardising Nutrient Gelatine.—In the finished medium it has been found experimentally that every cubic centimetre of meat extract has increased in bulk to 1.008 c.c. for every 0.01 gramme of added gelatine, and this factor must be taken into account in calculating the amount of soda solution necessary to produce the standard reaction in the finished medium.

The following expansion table shows at a glance the increase in bulk for the varying percentages:

GELATINE EXPANSION TABLE.

AT 20° CENTIGRADE.

100 c.c. meat extract		1000 c.c. meat extract	
+ 5 gms. gelatine	measure 104.0 c.c.	+ 50 gms. gelatine	measure 1040 c.c.
+ 6 " " "	104.8 "	+ 60 " " "	1048 "
+ 7 " " "	105.6 "	+ 70 " " "	1056 "
+ 8 " " "	106.4 "	+ 80 " " "	1064 "
+ 9 " " "	107.2 "	+ 90 " " "	1072 "
+10 " " "	108.0 "	+100 " " "	1080 "
+11 " " "	108.8 "	+110 " " "	1088 "
+12 " " "	109.6 "	+120 " " "	1096 "
+13 " " "	110.4 "	+130 " " "	1104 "
+14 " " "	111.2 "	+140 " " "	1112 "
+15 " " "	112.0 "	+150 " " "	1120 "
+20 " " "	116.0 "	+200 " " "	1160 "

The method of standardising gelatine is very similar to that described under bouillon.

For example, 1000 c.c. of meat extract are employed in the manufacture of 11 per cent. gelatine.

By referring to the above table it is found that after solution the medium mass measures 1088 c.c., and if three titrations are performed there still remain 1013 c.c. of medium to be standardised (instead of only 925 c.c.).

If the initial reaction of the gelatine mass is +24, it will require the addition of 14 c.c. $\frac{n}{1}$ NaOH per litre to render the reaction of the finished medium +10.

Therefore, 1000 c.c. : 1013 c.c. :: 14 c.c. : x.

$x = 14.182$ c.c. $\frac{n}{1}$ NaOH.

This quantity is replaced by

1.41 c.c.	dekanormal soda solution	(equivalent to 14.100 $\frac{n}{1}$ NaOH)
0.08 "	normal soda solution	(" " 0.080 $\frac{n}{1}$ NaOH)
0.02 "	decinormal soda solution	(" " 0.002 $\frac{n}{1}$ NaOH)
		14.182

giving the requisite weight of soda in a little over 1.5 c.c. of fluid.

Standardising Nutrient Agar.—Every cubic centimetre of meat extract increases in bulk to 1.0053 c.c. for every 0.01 gramme of added agar.

The following table gives the calculated bulk for varying percentage of agar:

AGAR EXPANSION TABLE.

At 20° Centigrade.

100 c.c. meat extract		1000 c.c. meat extract	
+0.5 gm. agar	measures 100.265 c.c.	+ 5 agar	measures 1002.65 c.c.
+1.0 " "	" 100.530 "	+10 "	" 1005.30 "
+1.5 gms. "	" 100.795 "	+15 "	" 1007.95 "
+2.0 " "	" 101.060 "	+20 "	" 1010.60 "

The method of standardising nutrient agar is similar to that described under nutrient gelatine, and a reference to the example there given will at once explain the necessary steps in the calculation.

THE FILTRATION OF MEDIA.

Fluid media are usually filtered through stout Swedish filter paper (occasionally through a porcelain filter candle), and in order to accelerate the rate of filtration the filter paper should be folded in that form which is known as the "physiological filter," not in the ordinary "quadrant" shape, as by this means a large surface is available for filtration and a smaller area in contact with the glass funnel supporting it.

To fold the filter proceed thus:

1. Take a circular piece of filter paper and fold it exactly through its centre to form a semicircle.

2. Fold the semicircle exactly in half to form a quadrant; make the crease distinct by running the thumbnail along it, then open the filter out to a semicircle again.

3. Fold each end of the semicircle in to the centre and so form another quadrant; smooth down the two new creases thus formed and again open out to a semicircle.

4. The semicircle now appears as in figure 82, *a*, the dark lines indicating the creases already formed.

5. Fold the point 1 over to the point 3, and 1*a* to 3*a*, to form the creases 4 and 4*a*, indicated in the diagram by the light lines. Fold point 1 over to 3, and 1*a* to 3*a*, to form the creases 5 and 5*a*.

6. Thus far the creases have all been made on the same side of the paper. Now subdivide each of the eight sectors by a crease through its centre on the opposite side of the paper, indicated by the broken line in the diagram. Fold up the filter gradually as each crease is made, and when finished the filter has assumed the shape of a wedge, as in figure 82, *b*.

When opened out the filter assumes the shape represented in figure 82, *c*.

The folded filter is next placed inside a glass funnel

supported on a retort stand and moistened with hot distilled water before the filtration of the medium is commenced.

Solid media are filtered through a specially made filter paper,—"papier Chardin,"—which is sold in boxes of twenty-five ready-folded filters.

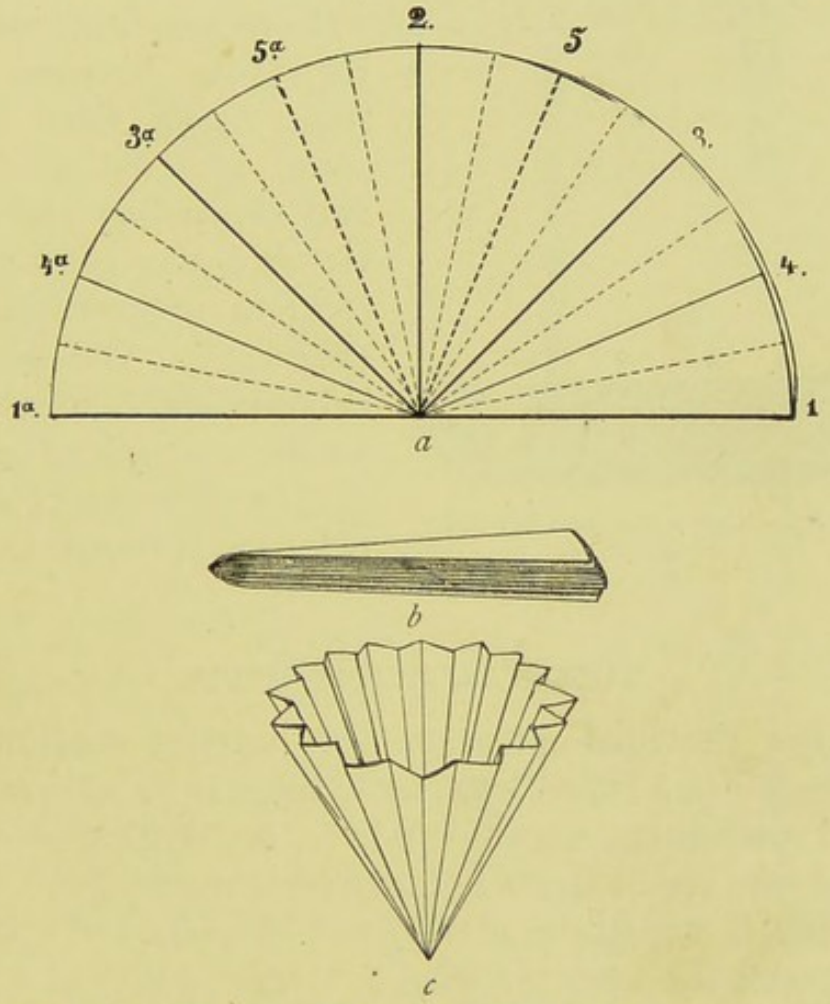


Fig. 82.—Filter folding: *a*, Filter folded in half, showing creases; *b*, appearance of filter on completion of folding; *c*, filter opened out ready for use.

Gelatine, when properly made, filters through this paper as quickly as bouillon does through the Swedish filter paper, and does *not* require the use of the hot-water funnel.

Agar, likewise, if properly made, filters readily, although not at so rapid a rate as gelatine. If badly

"egged," and also during the winter months, it is necessary to surround the glass funnel, in which the filtration of the agar is carried on, by a hot-water jacket. This is done by placing the glass funnel inside a double-walled copper funnel—the space between the walls being filled with water at about 90° C.—and supporting the latter on a ring gas burner fixed to a retort stand (Fig. 83). The gas is lighted and the water jacket maintained at a high temperature until filtration is completed.

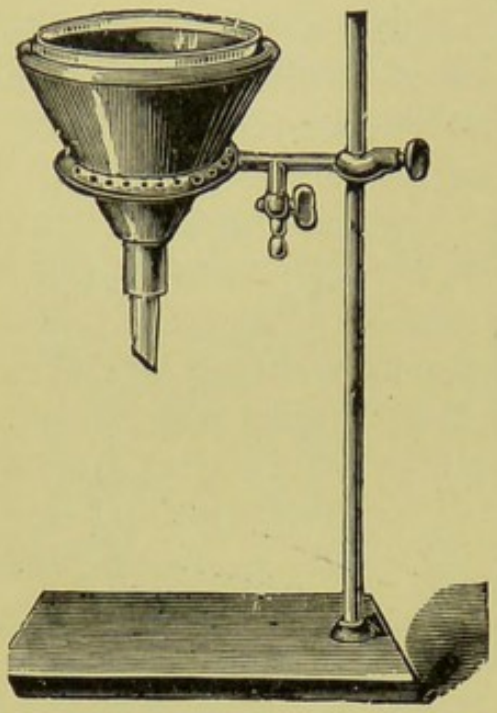


Fig. 83.—Ring burner and hot-water filter.

TUBING NUTRIENT MEDIA.

After the final filtration, the nutrient medium is "tubed"—*i. e.*, filled into sterile tubes in definite measured quantities, usually 10 c.c.; or "flasked"—*i. e.*, filled into sterile flasks in fairly large quantities. This process is sometimes carried out by means of a large separator funnel fitted with a "three-way" tap which communicates with a small graduated tube (capacity 20 c.c. and graduated in cubic centimetres) attached to the side. The shape of this piece of apparatus, known as Treskow's funnel, renders it particularly liable to damage. It is better, therefore, to arrange a less expensive piece of apparatus which will serve the purpose equally well (Fig. 84).

A Geissler's three-way stop-cock has the tube on one side of the tap ground obliquely at its extremity,

the tube on the opposite side cut off within 3 c.c. of the tap. The short tube is connected by means of a perforated rubber cork with a 10 c.c. length of stout glass tubing (1.5 c.c. bore). The third channel of the three-way tap is connected, by means of rubber tubing, with the nozzle of an ordinary separator funnel. Finally, the receiving cylinder above the three-way tap is graduated in cubic centimetres up to 20, by pouring into it

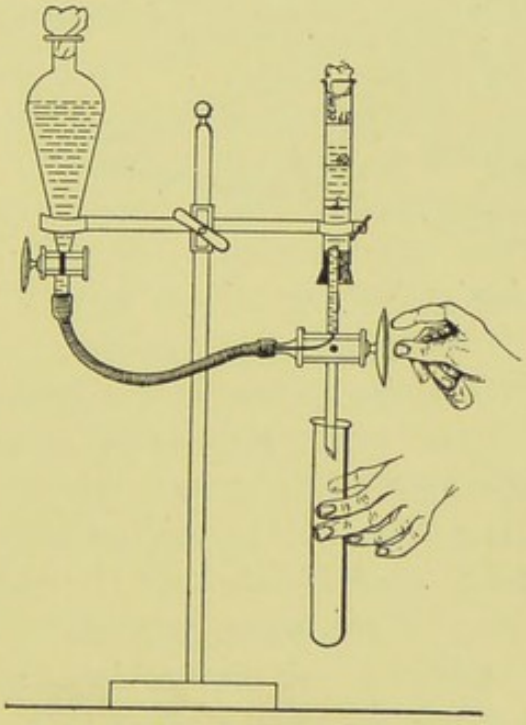


Fig. 84.—Three-way tap, home-made.

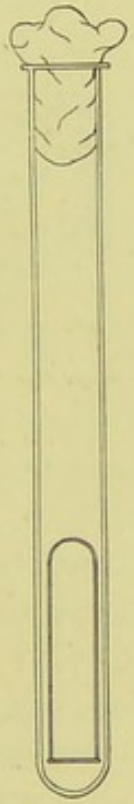


Fig. 85.—Gas tube (Durham).

measured quantities of water and marking the various levels on the outside with a writing diamond.

Fluid media containing carbohydrates are filled into fermentation tubes (*vide* Fig. 14); or into ordinary media tubes which already have smaller tubes, inverted, inside them (Fig. 85), to collect the products of growth of gas-forming bacteria. When first filled, the small tubes float on the surface of the medium; after the first sterilisation nearly all the air is replaced by the medium,

and after the final sterilisation the gas tubes will be submerged and completely filled with the medium.

Storing "Tubed" Media.—Media after being tubed are best stored by packing, in the vertical position, in oblong boxes having an internal measurement of 37 cm. long by 12 cm. wide by 10 cm. deep. Each box (Fig. 86) has a movable partition formed by the vertical face of a weighted triangular block of wood, sliding free on the bottom (Fig. 86, A); or by a flat

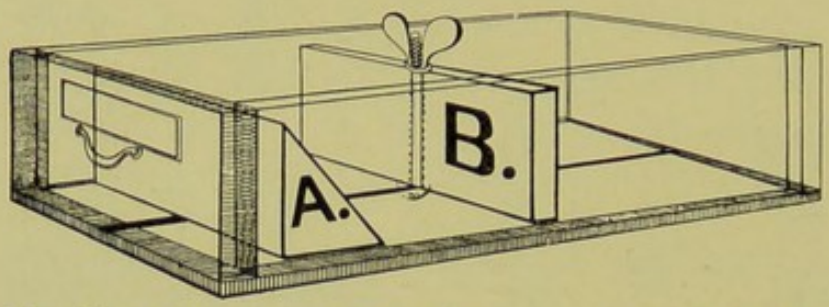


Fig. 86.—Medium box, showing alternative partitions A and B.

piece of wood sliding in a metal groove in the bottom of the box, which can be fixed at any spot by tightening the thumbscrew of a brass guide rod which transfixes the partition (Fig. 86, B). The front of the box is provided with a handle and a celluloid label for the name of the contained medium. These boxes are arranged upon shelves in a dark cupboard,—or preferably an iron safe,—which should be rendered as nearly air-tight as possible, and should have the words "media stores" painted on its doors.

X. CULTURE MEDIA.

Nutrient Bouillon.—

1. Measure 800 c.c. of meat extract into a litre flask.

2. Weigh out Witté's peptone, 10 grammes (= 1 per cent.), salt, 5 grammes (= 0.5 per cent.), and mix into a smooth paste with 200 c.c. of meat extract previously heated to 60° C. (Be careful to leave no unbroken globular masses of peptone.)

3. Add the peptone emulsion to the meat extract in the flask and heat the steamer for forty-five minutes (to completely dissolve the peptone, and to render the acidity of the meat extract stable).

4. Estimate the reaction of the medium; control the result; render the reaction of the finished medium +10 (*vide* page 133).

5. Heat for half an hour in the steamer at 100° C. (to complete the precipitation of the phosphates, etc.).

6. Filter through Swedish filter paper into a sterile flask.

7. Fill into sterile tubes (10 c.c. in each tube).

8. Sterilise in the steamer for twenty minutes on each of three consecutive days—*i. e.*, by the discontinuous method (*vide* page 41).

Inosite-free Media Bouillon (Durham).—

1. Prepare meat extract, 1000 c.c. (*vide* page 127), from bullock's heart which has been "hung" for a couple of days.

2. Prepare nutrient bouillon (+10), 1000 c.c. (*vide supra*), from the meat extract, and store in 1-litre flask.

3. Inoculate the bouillon from a pure cultivation of the B. lactis aerogenes, and incubate at 37° C. for forty-eight hours.

4. Heat in the steamer at 100° C. for twenty minutes to destroy the bacilli and some of their products.

5. Estimate the reaction of the medium and if necessary restore to +10.

6. Inoculate the bouillon from a pure cultivation of the B. coli communis and incubate at 37° C. for forty-eight hours.

7. Heat in the steamer at 100° C. for twenty minutes.

Now fill two fermentation tubes with the bouillon, tint with litmus solution, and sterilise; inoculate with B. lactis aerogenes. If no acid or gas is formed, the bouillon is in a sugar-free condition; but if acid or gas is present, again make the bouillon in the flask +10, reinoculate with one or other of the above-mentioned bacteria, and incubate; then test again. Repeat this till neither acid nor gas appears in the medium.

8. After the final heating, stand the flask in a cool place and allow the growth to sediment. Filter the supernatant broth through Swedish filter paper. If the filtrate is cloudy, filter through a porcelain filter candle.

9. Tube, and sterilise as for bouillon.

Bouillon prepared in the above-described manner will prove to be absolutely sugar-free; and from it may be prepared nutrient sugar-free gelatine or agar, by following the directions given on pages 145 and 149, respectively, substituting the inosite-free meat extract for the ordinary meat extract. The most important application of inosite-free bouillon is its use in the preparation of sugar bouillons, whether glucose, maltose, lactose, or saccharose, of exact percentage composition.

Glycerine Bouillon.—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Measure out glycerine, 60 c.c. (= 6 per cent.), and add to the bouillon.

3. Tube, and sterilise as for bouillon.

Sugar Bouillon.—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Weigh out glucose (anhydrous), 20 grammes (= 2 per cent.), and dissolve in the fluid.

3. Tube, and sterilise as for bouillon.

Ordinary commercial glucose serves the purpose equally well, but is not recommended, as during the process of sterilisation the medium gradually deepens in colour.

NOTE.—In certain cases a corresponding percentage of lactose, maltose, or saccharose is substituted for glucose.

Glucose Formate Bouillon (Kitasato).—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Weigh out glucose, 20 grammes (= 2 per cent.), sodium formate, 4 grammes (= 0.4 per cent.), and dissolve in the fluid.

3. Tube, and sterilise as for bouillon.

Sulphindigotate Bouillon (Weyl).—

1. Measure out nutrient bouillon (*vide* page 141, sections 1 to 6).

2. Weigh out glucose, 20 grammes (= 2 per cent.), sodium sulphindigotate, 1 gramme (= 0.1 per cent.), and dissolve in the fluid.

3. Tube, and sterilise as for bouillon.

NOTE.—The finished medium is of a blue colour, which during the growth of anaerobic bacteria is oxidised and decolourised to a light yellow.

Nitrate Bouillon.—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Weigh out potassium nitrate, 5 grammes (= 0.5 per cent.), and dissolve it in the bouillon.

3. Tube, and sterilise as for bouillon.

NOTE.—The nitrate of sodium or ammonium may be substituted for that of potassium, or the salt may be added in the proportion of from 0.1 to 1 per cent. to meet special requirements.

Iron Bouillon.—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Weigh out ferric tartrate, 1 gramme (= 0.1 per cent.), and dissolve it in the bouillon.

3. Tube, and sterilise as for bouillon.

N. B.—The lactate of iron may be substituted for the tartrate.

Lead Bouillon.—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Weigh out lead acetate, 1 gramme (= 0.1 per cent.), and dissolve it in the bouillon.

3. Tube, and sterilise as for bouillon.

Litmus Bouillon.—

1. Measure out nutrient bouillon, 1000 c.c. (*vide* page 141, sections 1 to 6).

2. Add sufficient sterile litmus solution to tint the medium a dark lavender colour. (Media rendered +10 will usually react very faintly alkaline or occasionally neutral to litmus.)

3. Tube, and sterilise as for bouillon.

Lactose Litmus Bouillon (Lakmus Molke).—

1. Weigh out peptone, 4 grammes, and emulsify it with 200 c.c. meat extract (*vide* page 127), previously heated to 60° C.

2. Weigh out salt, 2 grammes, and lactose, 20, and mix with the emulsion.

3. Wash the mixture into a sterile litre flask with 200 c.c. meat extract and add 600 c.c. distilled water.

4. Heat in the steamer at 100° C. for thirty minutes, to completely dissolve the peptone, etc.

5. Neutralise carefully to litmus paper by the successive additions of small quantities of decinormal soda solution.

6. Replace in the steamer for twenty minutes to precipitate phosphates, etc.

7. Filter through two thicknesses of Swedish filter paper.

8. Add sterile litmus solution, sufficient to colour the medium a deep purple.

9. Tube, and sterilise as for bouillon.

Parietti's Bouillon.—

1. Measure out pure hydrochloric acid, 4 c.c., and add to it carbolic acid solution (5 per cent.), 100 c.c. Allow the solution to stand at least a few days before use.

2. This solution is added in quantities of 0.1, 0.2, and 0.3 c.c. (delivered by means of a sterile graduated pipette) to tubes each containing 10 c.c. of previously sterilised nutrient bouillon (*vide* page 141).

3. Incubate at 37° C. for forty-eight hours to eliminate contaminated tubes. Store the remainder for future use.

Carbolised Bouillon.—

1. Prepare nutrient bouillon (*vide* page 141, sections 1 to 6).

2. Weigh out carbolic acid, 1 gramme (2.5 or 5 grammes may be needed for special purposes), and dissolve it in the medium.

3. Tube, and sterilise as for bouillon.

Nutrient Gelatine.—

1. Measure out meat extract, 800 c.c., into a 2-litre flask.

2. Weigh out Witté's peptone, 10 grammes (= 1 per cent.), salt, 5 grammes (= 0.5 per cent.), and mix into a smooth paste with 200 c.c. meat extract previously heated to 60° C.; add the emulsion to the meat extract in the flask.

3. Weigh out that quantity of best Gold Label French gelatine necessary to secure the required percentage,—usually 90 to 120 grammes (= 9 per cent. to 12 per cent.),—cut the sheets into small pieces and add to the meat extract.

4. Place the flask in the steamer at 100° C. for one hour.

5. Estimate the reaction of the medium mass and control the result; then add sufficient soda solution to render the reaction of the calculated bulk of medium +10. (See Gelatine Expansion Table, page 134.)

6. Replace in the steamer for twenty minutes (to complete the precipitation of the phosphates).

7. Allow the medium mass to cool to 60° C. Well whip the whites of two eggs, add to the contents of the flask, and replace in the steamer at 100° C. for about half an hour (until the egg-albumen has coagulated and formed large, firm masses floating on and in clear gelatine).

8. Filter through papier Chardin into a sterile flask.

9. Tube in quantities of 10 c.c.

10. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days—*i. e.*, by the discontinuous method.

Rapid Method of Preparing Nutrient Gelatine.—

1. Finely mince 500 grammes of lean beef and add to 800 c.c. of distilled water in a flask; place the flask in a water-bath, and raise the temperature of its contents to and keep at 45° C. for twenty minutes; then

rapidly raise the temperature to 100° C., and maintain there for ten minutes.

2. Weigh a 2-litre flask on a trip balance (Fig. 87) and note the weight, or counterpoise carefully. Filter

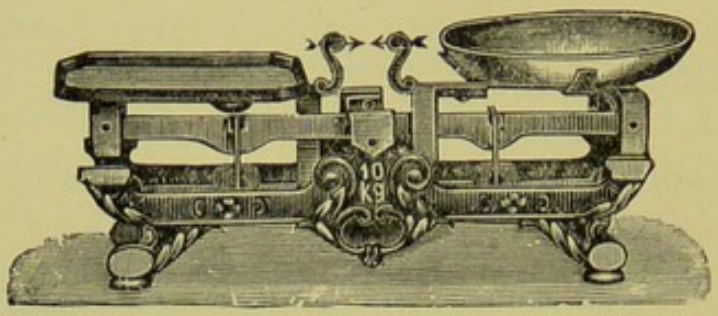


Fig. 87.—Trip balance.

the mixture into the flask. Do not make up the filtrate to 1000 c.c. as in the ordinary method.

An extremely useful counterpoise is a small sheet-brass cylinder about 38 mm. high and 38 mm. in diameter, with a funnel-shaped top and provided with a side tube by which its contents, fine "dust" shot, may be emptied out (Fig. 88).

3. Weigh out and mix 10 grammes of peptone, 5 grammes of salt, and make into a thick paste with 150 c.c. distilled water; then add the emulsion to the meat extract in the flask; also add 100 grammes sheet gelatine cut into small pieces, and return the flask to the water-bath.

Fig. 88. — Counterpoise; weight when empty, 35 grammes; when full of dust shot, 200 grammes.

4. Arrange a 10-litre tin can (with copper bottom, such as is used in the preparation of distilled water) by its side, fill the can with boiling water and place a lighted Bunsen burner under it. Fit a long safety tube to the neck of the can and also a delivery tube, bent twice at right angles, adjusted to reach to the bottom of the interior of the flask (Fig. 89).

5. Keep the water in the can vigourously boiling, and so steam at 100° C., bubbling through the medium mass, for ten minutes, by which time complete solution of the gelatine is effected.

6. Weigh the flask and its contents; then (1115[1] grammes + weight of the flask) minus (weight of the

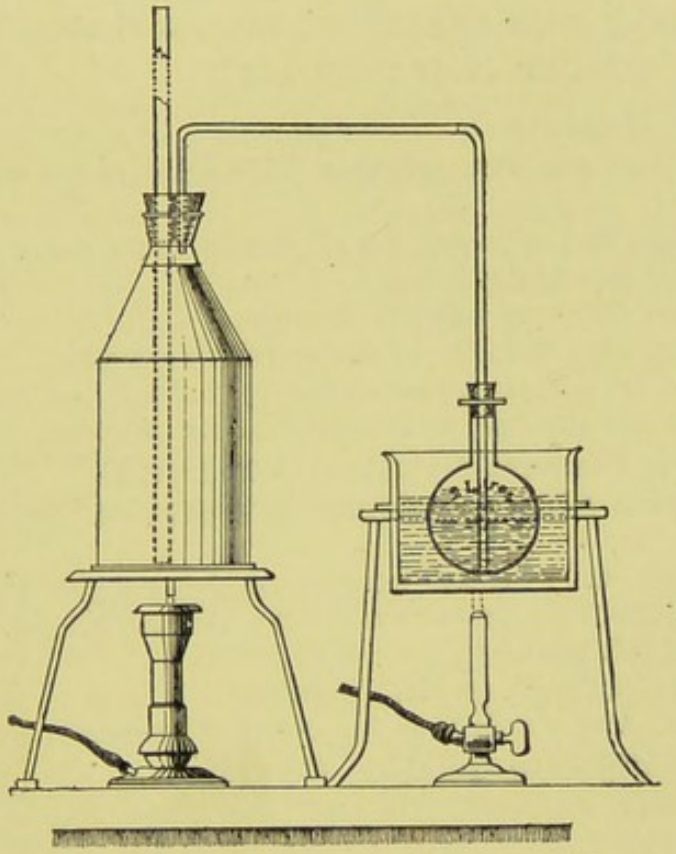


Fig. 89.—Steam can arrangement for media.

flask and its contents) equals the weight of water required to make up the bulk to 1 litre. Add the requisite quantity of water at the boiling-point.

7. Titrate and estimate the reaction of the medium

[1] This figure is obtained by adding together 1 litre water, 1000 grammes; 10 per cent. gelatine, 100 grammes; 1 per cent. peptone, 10 grammes; 0.5 per cent. salt, 5 grammes; total, 1115 grammes. Modifications of the above process, as to quantities and percentages, will require corresponding alterations of the figures. The average weight of 1 litre of 10 per cent. nutrient gelatine when prepared in this way is 1080 grammes (compare expansion table, page 134).

mass; control the result. Calculate the amount of soda solution required to make the reaction of the medium mass +10 (*i. e.*, calculate for 1000 c.c., less the quantity used for the titrations).

8. Add the necessary amount of soda solution and heat in the steamer at 100° C. for twenty minutes, to precipitate the phosphates, etc.

9. Clarify with egg; filter, tube, and sterilise as for nutrient gelatine (*vide* page 145).

Sugar Gelatine.—

1. Prepare nutrient gelatine (*vide* page 145, sections 1 to 7).

2. Weigh out glucose, 20 grammes (= 2 per cent.), and dissolve in the hot gelatine.

3. Filter through papier Chardin.

4. Tube, and sterilise as for nutrient gelatine.

NOTE.—In certain cases, lactose, maltose, or saccharose, in similar percentage, is substituted for glucose.

Glucose Formate Gelatine (Kitasato).—

1. Prepare nutrient gelatine (*vide* page 145, sections 1 to 7).

2. Weigh out glucose, 20 grammes (= 2 per cent.), and sodium formate, 4 grammes (= 0.4 per cent.), and dissolve in the hot gelatine.

3. Filter through papier Chardin.

4. Tube, and sterilise as for nutrient gelatine.

Sulphindigotate Gelatine (Weyl).—

1. Prepare nutrient gelatine (*vide* page 145, sections 1 to 7).

2. Weigh out glucose, 20 grammes (= 2 per cent.), and sodium sulphindigotate, 1 gramme (= 0.1 per cent.), and dissolve in the hot gelatine.

3. Filter through papier Chardin.

4. Tube, and sterilise as for nutrient gelatine.

Litmus Gelatine.—

1. Prepare nutrient gelatine (*vide* page 145, sections 1 to 8).

2. Add sterile litmus solution, sufficient to tint the medium a deep lavender colour.

3. Tube, and sterilise as for nutrient gelatine.

Lactose Litmus Gelatine (Wurtz).—

1. Prepare nutrient gelatine (*vide* page 145, sections 1 to 4).

2. Render the reaction of the medium mass —5.

3. Replace in the steamer at 100° C. for twenty minutes.
4. Clarify with egg as for gelatine.
5. Weigh out lactose, 20 grammes (= 2 per cent.), and dissolve it in the medium.
6. Filter through papier Chardin.
7. Add sufficient sterile litmus solution to colour the medium pale lavender.
8. Tube, and sterilise as for nutrient gelatine.

Carbolised Gelatine.—

1. Prepare nutrient gelatine (*vide* page 145, sections 1 to 7).
2. Weigh out carbolic acid, 5 grammes (= 0.5 per cent.), and dissolve it in the gelatine.
3. Filter through papier Chardin.
4. Tube, and sterilise as for nutrient gelatine.

One or 2.5 grammes of carbolic acid (= 0.1 per cent. or 0.25 per cent.) are occasionally used in place of the 5 grammes to meet special requirements.

Nutrient Agar-agar.—

1. Measure out meat extract, 600 c.c., into a 2-litre flask.

2. Weigh out Witté's peptone, 10 grammes (= 1 per cent.), salt, 5 grammes (= 0.5 per cent.), and mix them into a smooth paste with 200 c.c. meat extract previously heated to 60° C.; add the emulsion to the meat extract in the flask.

3. Weigh out that quantity of powdered agar necessary to secure the required percentage,—usually 15 to 20 grammes (= 1.5 to 2 per cent.),—and mix it into a smooth paste with 200 c.c. of meat extract (which must be quite cold, as the agar powder undergoes considerable expansion when first mixed with hot fluids and before solution takes place); add the emulsion to the meat extract, etc., in the flask.

4. Place the flask in the steamer at 100° C. until the agar is completely dissolved. This will take about ninety minutes.

5. Estimate the reaction of the medium mass; control the result; then add sufficient soda solution to

render the reaction of the calculated bulk of medium +10. (See Agar Expansion Table, page 135.)

6. Replace in the steamer for twenty minutes (to complete the precipitation of the phosphates, etc.).

7. Allow the medium mass to cool to 60° C. Well whip the whites of two eggs, add to the contents of the flask, and replace in the steamer at 100° C. for about half an hour (until the egg-albumen has coagulated and formed large, firm masses floating on and in clear agar).

8. Filter through papier Chardin, by the aid of a hot-water funnel (Fig. 83) into a sterile flask.

9. Tube, and sterilise in the steamer at 100° C. for thirty minutes on each of three consecutive days—*i. e.*, by the discontinuous method.

Brain Agar; Spleen Agar.—Use meat extract prepared from sheep (or ox) brain and spleen respectively, and proceed exactly as if making nutrient agar (*vide supra*).

Rapid Method of Preparing Nutrient Agar.—

1. Finely mince 500 grammes of lean beef and add to 800 c.c. of distilled water in a flask; place the flask in a water-bath, and raise the temperature of its contents to and keep at 45° C. for twenty minutes; then rapidly raise the temperature to 100° C., and maintain there for ten minutes.

2. Weigh a 2-litre flask and note the weight—or counterpoise exactly. Filter the mixture into the flask and again weigh, but do not make up the filtrate to 1000 c.c. as in the ordinary method.

3. Weigh out and mix 10 grammes of peptoné, 5 grammes of salt, and 20 grammes of powdered agar, and make into a thick paste with 150 c.c. distilled water, and add to the meat extract in the flask; return the flask to the water-bath.

4. Arrange a 10-litre tin can (with copper bottom, such as is used in the preparation of distilled water)

by its side, fill the can with boiling water, and place a lighted Bunsen burner under it. Fit a long safety tube to the neck of the can; also a delivery tube, bent twice at right angles and adjusted to reach to the bottom of the interior of the flask. (See also Rapid Method of Preparing Gelatine, page 145.)

5. Keep the water in the can vigourously boiling, and so steam at 100° C., bubbling through the medium mass, for twenty-five minutes, by which time complete solution of the agar is effected.

6. Now weigh the flask and its contents; then (1035 [1] grammes + weight of flask) minus (weight of flask and its contents) equals the weight of water required to make up the bulk of the medium to 1 litre. Add the requisite amount.

7. Titrate, and estimate the reaction of the medium mass; control the result. Calculate the amount of soda solution required to make the reaction of the medium mass +10 (*i. e.*, calculated for 1000 c.c., less the quantity used for the titrations).

8. Add the necessary amount of soda solution and heat in the steamer at 100° C. for twenty minutes.

9. Clarify with egg, filter, tube, and sterilise as for nutrient agar (*vide* page 150).

Glycerine Agar.—

1. Prepare nutrient agar (*vide* page 149, sections 1 to 8). Measure out 1000 c.c.

2. Measure out pure glycerine, 60 c.c. (= 6 per cent.), and add to the agar.

3. Tube, and sterilise as for nutrient agar.

Sugar Agar.—

1. Prepare nutrient agar (*vide* page 149, sections 1 to 8). Measure out 1000 c.c.

2. Weigh out glucose, 20 grammes (= 2 per cent.), and dissolve in the clear agar.

3. Tube, and sterilise as for nutrient agar.

NOTE.—In certain cases, lactose, maltose, or saccharose (in similar percentage) is substituted for glucose.

[1] Compare note on page 147. The average weight of 1 litre of 2 per cent. nutrient agar when completed is 1010.5 grammes.

Glucose Formate Agar (Kitasato).—

1. Prepare nutrient agar (*vide* page 149, sections 1 to 8). Measure out 1000 c.c.

2. Weigh out glucose, 20 grammes (= 2 per cent.), sodium formate, 4 grammes (= 0.4 per cent.), and dissolve in the agar.

3. Tube, and sterilise as for nutrient agar.

Sulphindigotate Agar.—

1. Prepare nutrient agar (*vide* page 149, sections 1 to 8). Measure out 1000 c.c.

2. Weigh out glucose, 20 grammes (= 2 per cent.), sodium sulphindigotate, 1 gramme (= 0.1 per cent.), and dissolve in the hot agar.

3. Tube, and sterilise as for nutrient agar.

Lactose Litmus Agar (Wurtz).—

1. Prepare nutrient agar (*vide* page 149, sections 1 to 4).

2. Render the reaction of the medium mass —5.

3. Replace in the steamer at 100° C. for twenty minutes.

4. Cool to 60° C. and clarify with egg as for nutrient agar.

5. Weigh out lactose, 20 grammes (= 2 per cent.), and dissolve it in the medium.

6. Filter through papier Chardin, using the hot-water funnel.

7. Add sterile litmus solution, sufficient to colour the medium a pale lavender.

8. Tube, and sterilise as for nutrient agar.

Carbolised Agar.—

1. Prepare nutrient agar (*vide* page 149, sections 1 to 8). Measure out 900 c.c.

2. Weigh out 1 gramme pure phenol and dissolve in the medium.

3. Tube, and sterilise as for nutrient agar.

Gelatine Agar.—This medium is prepared by adding to nutrient gelatine sufficient agar to ensure the solidity of the medium when incubated at temperatures above 22° C. If it is intended to employ an incubating temperature of 30° C., 10 per cent. gelatine and 0.5 per cent. agar must be dissolved in the meat extract before the addition of the peptone and salt; while for incubating at 37° C., 12 per cent. gelatine and 0.75 per cent. agar must be used. Avoid the addition of more agar than is absolutely necessary, otherwise the action upon the medium of such organisms as elaborate a liquefying ferment may be retarded or completely inhibited.

1. Measure out 600 c.c. meat extract into a 2-litre flask, and add to it gelatine, 100 grammes (or 120, as may be necessary).

2. Weigh out powdered agar, 5 grammes (or 7.5 grammes, as required), emulsify with 200 c.c. meat extract, and add to the contents of the flask.

3. Heat in the steamer at 100° C. for ninety minutes to completely dissolve the agar and gelatine.

4. Weigh out peptone, 10 grammes, salt, 5 grammes; emulsify with 200 c.c. meat extract previously heated to 60° C., and add to the contents of the flask.

5. Replace in the steamer for fifteen minutes.

6. Estimate the reaction; control the result. Then add sufficient caustic soda solution to render the reaction +10.

7. Replace in the steamer at 100° C. for twenty minutes.

8. Cool to 60° C. Clarify with egg as for nutrient gelatine.

9. Filter through papier Chardin, using the hot-water funnel.

10. Tube, and sterilise in the steamer at 100° C. for thirty minutes on each of three consecutive days.

Agar Gelatine (Guarniari).—

1. Measure out meat extract, 750 c.c., into a 2-litre flask, and add to it gelatine, 50 grammes.

2. Weigh out powdered agar, 3 grammes; emulsify with cold distilled water, 50 c.c., and add to the contents of the flask.

3. Heat in the steamer at 100° C. for sixty to ninety minutes to completely dissolve the agar and gelatine.

4. Weigh out Witté's peptone, 25 grammes, salt, 5 grammes, and emulsify with 200 c.c. meat extract previously heated to 60° C., and add to the contents of the flask.

5. Replace in the steamer for fifteen minutes.

6. Neutralise carefully to litmus paper by the successive additions of small quantities of normal soda solution.

7. Replace in the steamer at 100° C. for twenty minutes.

8. Cool to 60° C. Clarify with egg as for nutrient gelatine.

9. Filter through papier Chardin, using the hot-water funnel.

10. Tube, and sterilise in the steamer at 100° C. for thirty minutes on each of three consecutive days.

Blood-serum.—

1. Sterilise cylindrical glass jar (Fig. 90) and its cover by dry heat, or by washing first with ether and then with alcohol.

2. Collect blood from sheep or ox in the sterile cylinder.

3. Allow the vessel to stand for fifteen minutes for the blood to coagulate. (This must be done before leaving the slaughter-house, otherwise the serum will be stained with hæmoglobin.)

4. Separate the clot from the sides of the vessel by means of a sterile glass rod (the yield of serum is much smaller when this is not done), and place the cylinder in the ice-chest for twenty-four hours.

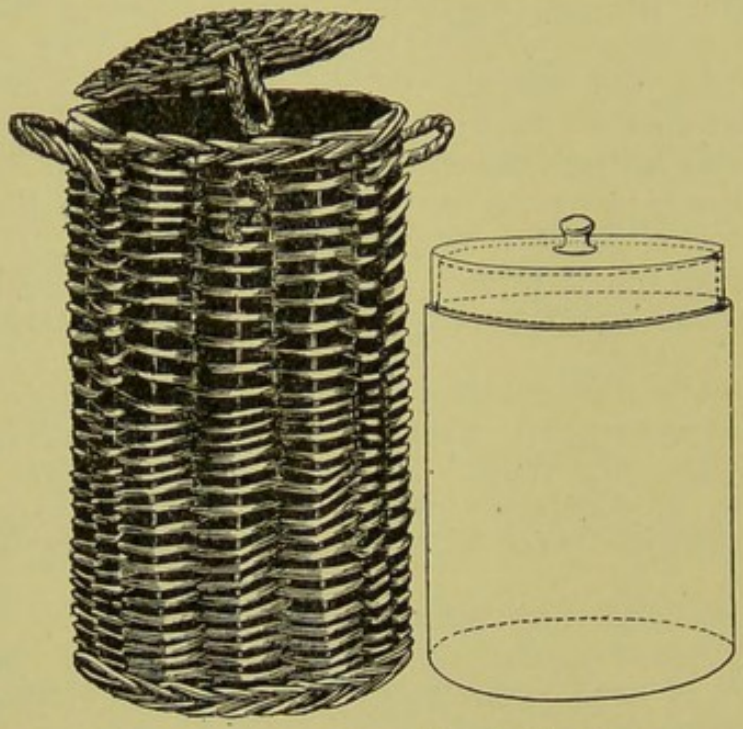


Fig. 90.—Blood-serum jar with wicker basket for transport.

5. Remove the serum with sterile pipettes, or syphon it off, and fill into sterile tubes (5 c.c. in each) or flasks.

6. Sterilise the serum by the fractional method—that is, by exposure in a water-bath to a temperature of 56° C. for half an hour on each of six consecutive days; store in the fluid condition.

6*a*. Or heat tubes containing serum to 56° C. in a water-bath for half an hour on each of two successive days.

7. On the third day, heat the tubes, in a sloping position, in a serum inspissator to about 72° C. (A coagulum is formed at this temperature which is fairly transparent; above 72° C., a thick turbid coagulum is formed.)

The serum inspissator (Fig. 91) in its simplest form is a double-walled rectangular copper box, closed in by a loose glass lid, and cased in felt or asbestos—the space between the walls is filled with water. The inspissator is supported on adjustable legs so that the

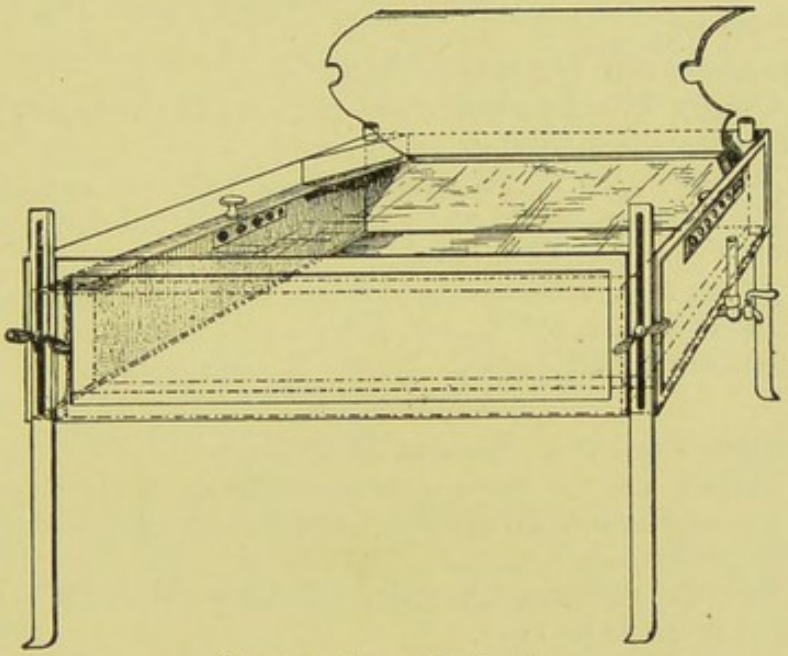


Fig. 91.—Serum inspissator.

serum may be solidified at any desired "slant," and is heated from below by a Bunsen burner. The more elaborate forms resemble the hot-air oven (Fig. 18) in shape and are provided with thermo-regulators.

8. Place the tubes in the incubator at 37° C. for forty-eight hours in order to eliminate those that have been contaminated. Store the remainder in a cool place for future use.

Glycerine Blood-serum.—

1. Prepare blood-serum as described above, sections 1 to 4.

2. Add 6 per cent. pure glycerine.

3. Complete as described above for ordinary blood-serum, sections 5 to 7.

NOTE.—Different percentages of glycerine—from 4 per cent. to 8 per cent.—are used for special purposes. Five per cent. is that usually employed.

Blood-serum (Löffler).—

1. Prepare nutrient bouillon (*vide* page 141), using meat extract made from veal instead of beef.

2. Add 1 per cent. glucose to the bouillon, and allow it to dissolve completely.

3. Now add 300 c.c. clear blood-serum (*vide* page 153, sections 1 to 4) to every 100 c.c. of this bouillon.

4. Fill into sterile tubes and complete as for ordinary blood-serum.

Blood-serum (Lorrain Smith).—

1. Collect blood-serum (*vide* page 153, sections 1 to 4), as free from hæmoglobin as possible.

2. Weigh out 0.15 per cent. sodium hydrate and dissolve it in the fluid (or add 0.375 c.c. of dekanormal soda solution for every 100 c.c. of serum).

3. Tube, and stiffen at 100° C. in the serum inspissator.

4. Incubate at 37° C. for forty-eight hours to eliminate any contaminated tubes. Store the remainder for future use.

Ascitic Bouillon (Serum Bouillon).—

1. Collect ascitic fluid (pleuritic fluid, hydrocele fluid, etc., by aspiration directly into sterile flasks, under strict aseptic precautions.

2. Mix the serum with twice its bulk of sterile nutrient bouillon (*vide* page 141).

3. If considered necessary (on account of the presence of blood, crystals, etc.), filter the serum bouillon through porcelain filter candle.

4. Tube, and sterilise by the fractional method for half an hour on each of five consecutive days.

5. Incubate at 37° C. for forty-eight hours and eliminate contaminated tubes. Store for future use.

Serum Agar (Wertheimer).—

1. Prepare nutrient agar (*vide* page 149), to the following formula: agar, 2 per cent.; peptone, 2 per cent.; salt, 0.5 per cent.; meat extract, *quantum sufficit.*

2. Make reaction of medium +10.

3. Filter; tube in quantities of 5 c.c.

4. Sterilise by the discontinuous method.

5. After the last sterilisation cool to 42° C., then add 5 c.c. sterile blood-serum from human placenta (sterilised,

if necessary, by the fractional method) to each tube; slope the tubes.

6. When set, incubate at 37° C. for forty-eight hours, to eliminate any contaminated tubes. Store the remainder for future use.

Serum Agar (Heiman).—

1. Prepare nutrient agar (*vide* page 149), to following formula: agar, 2 per cent.; peptone, 1.5 per cent.; salt, 0.5 per cent.; glucose, 2 per cent; meat extract, *quantum sufficit.*

2. Make reaction of medium +10.

3. Filter; tube in quantities of 6 c.c.

4. Sterilise by the discontinuous method.

5. After the third sterilisation cool the tubes to 42° C., and add to each 3 c.c. of sterile hydrocele fluid, ascitic fluid, or pleuritic effusion (previously sterilised, if necessary, by the fractional method); allow the tubes to solidify in a sloping position.

6. When solid, incubate at 37° C. for forty-eight hours, and eliminate any contaminated tubes. Store the remainder for future use.

Serum Agar (Kanthack and Stevens).—

1. Collect ascitic, pleuritic, or hydrocele fluid in sterile flasks and allow it to stand in the ice-chest for twelve hours to sediment.

2. Decant the clear fluid into a measuring cylinder.

3. Add 0.5 c.c. dekanormal NaOH solution for every 100 c.c. serum, and mix thoroughly.

4. Heat in the steamer for sixty minutes.

5. Weigh out 15 grammes agar, emulsify with about 200 c.c. of the alkaline fluid, and add to the remainder of the fluid.

6. Heat in the steamer ninety minutes to dissolve the agar.

7. Filter through papier Chardin, using a hot-water funnel.

8. Weigh out glucose, 1 per cent., and dissolve it in the clear agar.

8*a*. If desired, add glycerine, 5 per cent., to the clear agar.

9. Tube, and sterilise in the steamer at 100° C. for thirty minutes on each of three consecutive days.

Blood Agar (Washbourn).—

1. Melt up several tubes of nutrient agar (*vide* page 149) and allow them to solidify in the oblique position.

2. Place the tubes, in the horizontal position, in the "hot" incubator for forty-eight hours, to evaporate off some of the condensation water.

3. Kill a small rabbit with chloroform and nail it out on a board (as for a necropsy). Moisten the hair thoroughly with 2 per cent. solution of lysol.

4. Sterilise several pairs of forceps, scissors, etc., by boiling.

5. Reflect the skin over the thorax with sterile instruments.

6. Open the thoracic cavity by the aid of a fresh set of sterile instruments.

7. Open the pericardium with another set of sterile instruments.

8. Sear the surface of the left ventricle with a red-hot iron and remove fluid blood from the heart by means of sterile pipettes (*e. g.*, those shown in Fig. 10, *c*).

9. Deliver a small quantity of the blood on the slanted surface of the agar in each of the tubes, and allow it to run over the entire surface of the medium.

10. Place the tubes in the horizontal position and allow the blood to coagulate.

11. Return the "blood agar" to the hot incubator for forty-eight hours and eliminate any contaminated tubes. Store the remainder for future use.

Urine Media.—

1. Collect freshly passed urine in sterile flask or flasks.
2. Place the flask in the steamer at 100° C. for thirty minutes.
3. Filter through two thicknesses of Swedish filter paper.
4. Tube, and sterilise by the discontinuous method.

(Leave the reaction unaltered.)

Urine Gelatine.—

1. Collect freshly passed urine in sterile flask.

2. Take the specific gravity, and, if above 1010, dilute with sterile water until that point is reached.

3. Estimate (with control) at the boiling-point, and note the reaction of the urine.

4. Weigh out gelatine, 10 per cent., and add to the urine in the flask.

5. Heat in the steamer at 100° C. for one hour to dissolve the gelatine.

6. Estimate the reaction and add sufficient caustic soda solution to restore the reaction of the medium mass to the equivalent of the original urine.

7. Cool to 60° C. and clarify with egg as for nutrient gelatine (*vide* page 145).

8. Filter through papier Chardin.

9. Tube, and sterilise as for gelatine.

Urine Gelatine (Heller).—

1. Collect freshly passed urine in sterile flask.

2. Filter through animal charcoal to remove part of the colouring matter.

3. Take the specific gravity, and if above 1010, dilute with sterile water till this gravity is reached.

4. Add Witté's peptone, 1 per cent.; salt, 0.5 per cent.; gelatine, 10 per cent.

5. Heat in the steamer at 100° C. for one hour, to dissolve the gelatine, etc.

6. Add normal caustic soda solution in successive small quantities, and test the reaction from time to time with litmus paper, until the fluid reacts faintly alkaline.

7 Cool to 60° C. and clarify with egg as for nutrient gelatine (*vide* page 145).

8. Filter through papier Chardin.

9. Tube, and sterilise as for nutrient gelatine.

Urine Agar.—

1. Collect freshly passed urine in sterile flask.

2. Weigh out 1.5 per cent. or 2 per cent. powdered agar, and add it to the urine.

3. Heat in the steamer ninety minutes to dissolve the agar.

4. Cool to 60° C. and clarify with egg as for nutrient agar (*vide* page 150).

5. Filter through papier Chardin, using the hot-water funnel.

6. Tube, and sterilise as for nutrient agar.

(Leave the reaction unaltered.)

Egg-albumen.—

1. Break several fresh eggs (hens', ducks', or turkeys' eggs), and collect the "whites" in a graduated cylinder, taking care to avoid admixture with the yolks.

2. Add 40 per cent. distilled water, and incorporate the mixture thoroughly by the aid of an egg-whisk.

3. Weigh out 0.15 per cent. sodium hydrate and dissolve it in the fluid (or add the amount of dekanormal caustic soda solution calculated to yield the required percentage of soda in the total bulk of the fluid—*i. e.*, 0.375 c.c. of dekanormal NaOH solution per 100 c.c. of the mixture).

3*a*. Glucose to the extent of 1 to 2 per cent. may now be added, if desired.

4. Strain the mixture through butter muslin and filter through a porcelain filter candle into a sterile filter flask.

5. Tube, and stiffen at 100° C. in the serum inspissator.

6. Incubate at 37° C. for forty-eight hours and eliminate any contaminated tubes; store the remainder for future use.

Egg-albumen (Tarchanoff and Kolesnikoff).—

1. Place unbroken hens' eggs in dekanormal caustic soda solution for ten days. (After this time the white becomes firm like gelatine.)

2. Carefully remove the shell and cut the egg into fine slices.

3. Wash for two hours in running water.

4. Place the egg slices in a large beaker and sterilise in the steamer at 100° C. for one hour.

5. Transfer each slice of egg by means of a pair of sterilised forceps to a Petri dish or large capsule.

6. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

Milk.—

1. Pour 1 litre of fresh milk into a large separator funnel, and heat in the steamer at 100° C. for one hour.

2. Remove from the steamer and estimate the re-

action of the milk (normal cows' milk averages +17). If of higher acidity than +20, or lower than +10, reject this sample of milk and proceed with another litre of milk from a different source.

Carefully eliminate milk to which antiseptics have been added as preservatives.

3. Allow the milk to cool, when the fat or cream will rise to the surface and form a thick layer.

4. Draw off the subnatant fat-free milk into sterile tubes (10 c.c. in each).

5. Sterilise in the steamer at 100° C. for twenty minutes on each of five successive days.

6. Incubate at 37° C. for forty-eight hours and eliminate any contaminated tubes. Store the remainder for future use.

Litmus Milk.—

1. Prepare milk as described above, sections 1 to 3.

2. Draw off the fat-free milk into a flask.

3. Add sterile litmus solution, sufficient to colour the milk a deep lavender.

4. Tube, sterilise, etc., as for milk.

Litmus Whey.—

1. Curdle fresh milk by adding rennet (or by acidifying with hydrochloric acid).
2. Filter off the whey into a sterile flask.
3. Heat in the steamer at 100° C. for one hour.
4. Filter into a sterile flask.
5. Tint the whey with litmus solution to a deep purple red.
6. Tube, and sterilise as for milk.

Whey Gelatine.—

1. Curdle fresh milk by adding rennet; filter off the whey into a sterile flask.
2. Estimate and note the reaction of the whey.
3. Weigh out gelatine, 10 per cent., and add it to the whey in the flask.
4. Heat in the steamer at 100° C. for one hour to dissolve the gelatine.
5. Estimate the reaction of the medium mass; then add sufficient caustic soda solution to restore the reaction of the medium mass to the equivalent of the original whey.

6. Cool to 60° C. and clarify with egg as for nutrient gelatine (*vide* page 145).
7. Filter through papier Chardin.
8. Tube, and sterilise as for nutrient gelatine.

Whey Agar.—

1. Curdle fresh milk by adding rennet; filter off the whey into a sterile flask.
2. Weigh out agar, 1.5 or 2 per cent., and add it to the whey in the flask.
3. Heat in the steamer at 100° C. for one hour, to dissolve the agar.
4. Cool to 60° C.; clarify with egg as for nutrient agar (*vide* page 150).
5. Filter through papier Chardin, using the hot-water funnel.
6. Tube, and sterilise as for nutrient agar.

Fish Bouillon.—

1. Weigh out herring, mackerel, or cod, 500 grammes, and place in a large porcelain beaker (or enamelled iron pot).
2. Weigh out sodium chloride, 26.5 grammes; potassium chloride, 0.75 gramme; magnesium chloride, 3.25 grammes; and dissolve in 1000 c.c. distilled water. Add the solution to the fish in the beaker.
3. Place the beaker in a water-bath and proceed as if preparing meat extract—*i. e.*, heat gently at 40° C. for twenty minutes, then rapidly raise the temperature to, and maintain at, the boiling-point for ten minutes.
4. Strain the mixture through butter muslin into a clean flask.
5. Weigh out peptone, 5 grammes, and emulsify with about 200 c.c. of the hot fish water; incorporate thoroughly with the remainder of the fish water in the flask.
6. Heat in the steamer at 100° C. for twenty minutes to complete the solution of the peptone.
7. Filter through Swedish filter paper.
8. When the fish bouillon is cold, make up to 1000 c.c. by the addition of distilled water (to replace the loss from evaporation).

As an alternative method "Marvis" fish food (16 grammes) may be substituted for the 500 grammes of fresh fish.

Fish Gelatine.—

1. Measure out fish bouillon, 1000 c.c., into a 2-litre flask.
2. Add sheet gelatine, 100 grammes, cut into small pieces.
3. Heat in the steamer at 100° C. for one hour.
4. Cool to below 60° C., and clarify with egg.
5. Filter through papier Chardin.

6. Tube, and sterilise as for nutrient gelatine.

Shake well after the final sterilisation, to aerate the medium.

Fish Gelatine—Agar.—

1. Weigh out powdered agar, 5 grammes, and emulsify it with 200 c.c. fish bouillon.

2. Wash the emulsion into a 2-litre flask with 800 c.c. fish bouillon.

3. Weigh out sheet gelatine, 70 grammes, cut it into small pieces and add it to the contents of the flask.

4. Heat in the steamer at 100° C. for one hour.

5. Cool to below 60° C. and clarify with egg.

6. Filter through papier Chardin.

7. Tube, and sterilise as for fish gelatine.

Shake well after the final sterilisation, to aerate the medium.

Potato.—

1. Choose fairly large potatoes, wash them well, and scrub the peel with a stiff nail-brush.

2. Peel and take out the eyes.

3. Remove cylinders from the longest diameter of each potato by means of an apple-corer or a large cork-borer (*i. e.*, one of about 1.4 cm. diameter).

3*a*. The reaction of the fresh potato is strongly acid to phenolphthalein. If, therefore, the potatoes are required to approximate +10, as for the cultivation of some of the vibrios, the cylinders should be soaked in a 1 per cent. solution of sodium carbonate for thirty minutes.

4. Cut each cylinder obliquely from end to end, forming two wedge-shaped portions.

5. Place a small piece of sterilised cotton-wool, moistened with sterile water, at the bottom of a sterile test-tube; insert the potato wedge into the tube so that its base rests upon the cotton-wool. Now plug the tube with cotton-wool (Fig. 92).

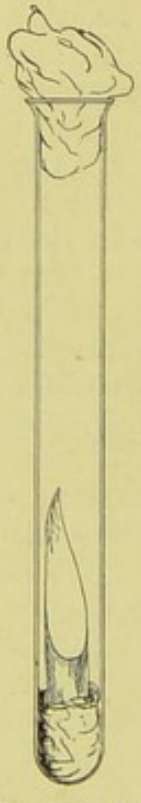


Fig. 92.—Potato tube.

6. Sterilise in the steamer at 100° C. for twenty minutes on each of *five* consecutive days.

Beet-root, carrot, turnip, and parsnip are prepared, tubed, and sterilised as potato.

Glycerinated Potato.—

1. Prepare ordinary potato wedges (*vide* page 163, sections 1 to 4).
2. Soak the wedges in 25 per cent. solution of glycerine for fifteen minutes.
3. Moisten the cotton-wool pads at the bottom of the potato tubes with a 25 per cent. solution of glycerine instead of plain water.
4. Insert a wedge of potato in each tube and replug the tubes.
5. Sterilise in the steamer at 100° C. for twenty minutes on each of *five* consecutive days.

Glycerine Potato Broth.—

1. Take 1 kilo of potatoes, wash thoroughly in water, peel, and grate finely on a bread-grater.
2. Weigh the potato gratings, place them in a 2-litre flask, and add distilled water in the proportion of 1 c.c. for every gramme weight of potato. Allow the flask to stand in the ice-chest for twelve hours.
3. Strain the mixture through butter muslin and filter through Swedish filter paper into a graduated cylinder. Note the amount of the filtrate.
4. Place the filtrate in a flask, add an equal quantity of distilled water, and heat in the steam steriliser for sixty minutes.
5. Add glycerine, 4 per cent., mix thoroughly, and again filter.
6. Tube and sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

Potato Gelatine (Elsner).—

1. Take 1 kilo of potatoes, wash thoroughly in water, peel, and finally grate finely on a bread-grater.
2. Weigh the potato gratings, place them in a 2-litre flask, and add distilled water in the proportion of 1 c.c. for every gramme weight of potato. Allow the flask to stand in the ice-chest for twelve hours.
3. Strain the mixture through butter muslin, and filter through Swedish filter paper into a graduated cylinder.
4. Add 15 per cent. gelatine to the potato decoction and heat in the steamer for sixty minutes.
5. Estimate the reaction and render the reaction of the medium mass +25.

6. Cool the medium to below 60° C.; clarify with egg as for nutrient gelatine (*vide* page 145).
7. Add 1 per cent. potassium iodide (powdered) to the medium.
8. Filter through papier Chardin.
9. Tube in quantities of 10 c.c.
10. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

Potato Gelatine (Goadby).—

1. Prepare glycerine potato broth as above, sections 1 to 5.
2. Add 10 per cent. gelatine to the potato decoction and heat in the steamer at 100° C. for sixty minutes.
3. Estimate the reaction and render +5.
4. Cool the medium to below 60° C., clarify with egg as for nutrient gelatine.
5. Filter through papier Chardin.
6. Tube, and sterilise as for nutrient gelatine.

Hay Infusion.—

1. Weigh out dried hay, 10 grammes, chop it up into fine particles and place in a flask.
2. Add 1000 c.c. distilled water, heated to 70° C.; close the flask with a solid rubber stopper.
3. Macerate in a water-bath at 60° C. for three hours.
4. Replace the stopper by a cotton-wool plug, and heat in the steamer at 100° C. for one hour.
5. Filter through Swedish filter paper.
6. Tube, and sterilise in the steamer at 100° C. for one hour on each of three consecutive days.

Beer Wort.—Wort is chiefly used as a medium for the cultivation of yeasts, etc., both in its fluid form and also when made solid by the addition of gelatine or agar. The wort is prepared as follows:

1. Weigh out 250 grammes crushed malt and place in a 2-litre flask.
2. Add 1000 c.c. distilled water, heated to 70° C., and close the flask with a rubber stopper.
3. Place the flask in a water-bath regulated to 60° C. and allow the maceration to continue for one hour.
4. Strain through butter muslin into a clean flask and heat in the steamer for thirty minutes.
5. Filter through Swedish filter paper.
6. Tube in quantities of 10 c.c. or store in flasks.

7. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

The natural reaction of the wort should *not* be interfered with.

NOTE.—It is sometimes more convenient to obtain "*unhopped*" [1] beer wort direct from the brewery. In this case it is diluted with an equal quantity of distilled water, steamed for an hour, filtered, filled into sterile flasks or tubes, and sterilised by the discontinuous method.

Wort Gelatine.—

1. Measure out wort (prepared as above), 1000 c.c., into a sterile flask.

2. Weigh out gelatine, 100 grammes (= 10 per cent.), and add it to the wort in the flask.

3. Heat in the steamer at 100° C. for one hour, to dissolve the gelatine.

4. Cool to 60° C.; clarify with egg as for nutrient gelatine (*vide* page 145).

5. Filter through papier Chardin.

6. Tube, and sterilise as for nutrient gelatine.

Wort Agar.—

1. Measure out wort (as above), 800 c.c., into a sterile flask.

2. Weigh out powdered agar, 20 grammes; mix into a smooth paste with 200 c.c. of cold wort and add to the wort in the flask.

3. Heat in the steamer at 100° C. for ninety minutes, to dissolve the agar.

4. Cool to 60° C.; clarify with egg as for nutrient agar (*vide* page 150).

5. Filter through papier Chardin, using the hot-water funnel.

6. Tube, and sterilise as for nutrient agar.

[1] "Hopped" wort exerts a toxic effect upon many bacteria, including the lactic acid bacteria.

Wine Must.—(Wine must is obtained from Sicily, in hermetically sealed tins, in a highly concentrated form,—as a thick syrup,—but not sterilised.)

1. Weigh out "wine must," 200 grammes, place in a 2-litre flask and add distilled water, 800 c.c.
2. Weigh out ammonium tartrate, 5 grammes, and add to the dilute must.
3. Place the flask in a water-bath regulated to 60° C. for one hour and incorporate the mixture thoroughly by frequent shaking.
4. Filter through papier Chardin.
5. Tube, and sterilise by the discontinuous method (three days).

Wheat Broth (Gasperini).—

1. Weigh out and mix wheat flour, 150 grammes; magnesium sulphate, 0.5 gramme; potassium nitrate, 1 gramme; glucose, 15 grammes.
2. Dissolve the mixture in 1000 c.c. of water heated to 100° C.
3. Filter through papier Chardin.
4. Tube, and sterilise by the discontinuous method.

Bread Paste.—

1. Grate stale bread finely on a bread-grater.
2. Distribute the crumbs in sterile Erlenmeyer flasks, sufficient to form a layer about half an inch thick over the bottom of each.
3. Add as much distilled water as the crumbs will soak up, but not enough to cover the bread.
4. Plug the flasks and sterilise in the steamer at 100° C. for thirty minutes on each of *four* consecutive days.

Milk Rice (Eisenberg).—

1. Measure out nutrient bouillon, 70 c.c., and milk, 210 c.c., and mix thoroughly.
2. Weigh out rice powder, 100 grammes, and rub it up in a mortar with the milk and broth mixture.
3. Fill the paste into sterile capsules, spreading it out so as to form a layer over the bottom of each.
4. Heat over a water-bath at 100° C. until the mixture solidifies.
5. Replace the lids of the capsules. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

(A solid medium of the colour of *café au lait* is thus produced.)

Milk Rice (Soyka).—

1. Measure out nutrient bouillon, 50 c.c., and milk, 150 c.c., and mix thoroughly.

2. Weigh out rice powder, 100 grammes, and rub it up in a mortar with the milk and broth mixture.

3. Fill the paste into sterile capsules, to form a layer over the bottom of each.

4. Replace the lids of the capsules.

5. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

(A pure white, opaque medium is thus formed.)

Peptone Water (Dunham).—

1. Weigh out Witté's peptone, 10 grammes, and salt, 5 grammes, and emulsify with about 250 c.c. of distilled water previously heated to 60° C.

2. Pour the emulsion into a litre flask and make up to 1000 c.c. by the addition of distilled water.

3. Heat in the steamer at 100° C. for thirty minutes.

4. Filter through Swedish filter paper.

5. Tube in quantities of 10 c.c. each.

6. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

Peptone Rosolic Acid Water.—

1. Weigh out rosolic acid (coralline), 0.5 gramme, and dissolve it in 80 per cent. alcohol, 100 c.c. Keep this as a stock solution.

2. Measure out peptone water (Dunham), 100 c.c., and rosolic acid solution, 2 c.c., and mix.

3. Heat in the steamer at 100° C. for thirty minutes.

4. Filter through Swedish filter paper.

5. Tube, and sterilise as for peptone water.

Iron Peptone Solution (Pakes).—

1. Weigh out peptone, 30 grammes, and emulsify it with 200 c.c. tap water, previously heated to about 60° C.

2. Wash the emulsion into a litre flask with 80 c.c. tap water.

3. Weigh out salt, 5 grammes, and sodium phosphate, 3 grammes, and dissolve in the mixture in the flask.

4. Heat the mixture in the steamer at 100° C. for thirty minutes, to complete the solution of the peptone, and filter into a clean flask.

5. Fill into tubes in quantities of 10 c.c. each.

6. Add to each tube 0.1 c.c. of a 2 per cent. neutral solu-

tion of ferric tartrate. (A yellowish-white precipitate forms.)

7. Sterilise as for peptone solution.

NOTE.—A similar quantity of a 1 per cent. neutral solution of lead acetate may be substituted for the iron salt.

Nitrate Water (Pakes).—

1. Weigh out Witté's peptone, 10 grammes, and emulsify it with 200 c.c. ammonia-free distilled water previously heated to 60° C.

2. Wash the emulsion into a flask and make up to 1000 c.c., with similar water.

3. Heat in the steamer at 100° C. for twenty minutes.

4. Weigh out sodium nitrate, 1 gramme, and dissolve in the contents of the flask.

5. Filter through Swedish filter paper.

6. Tube, and sterilise as for peptone water.

Bile Salt Broth (MacConkey).—

1. Weigh out Witté's peptone, 20 grammes (= 2 per cent.), and emulsify with 200 c.c. distilled water previously warmed to 60° C.

2. Weigh out sodium taurocholate (commercial), 5 grammes (= 0.5 per cent.), and glucose, 5 grammes (= 0.5 per cent.), and dissolve in the peptone emulsion.

3. Wash the peptone emulsion into a flask with 800 c.c. distilled water, and heat in the steamer at 100° C. for twenty minutes.

4. Filter through Swedish filter paper into a sterile flask.

5. Add sterile litmus solution sufficient to colour the medium to a deep purple.

6. Fill, in quantities of 10 c.c., into tubes containing small gas tubes (*vide* Fig. 85, page 139), and sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

Bile Salt Agar (MacConkey).—

1. Weigh out powdered agar, 15 grammes (= 1.5 per cent.), and emulsify with 200 c.c. *cold* distilled water.

2. Weigh out peptone, 20 grammes (= 2 per cent.), and emulsify with 200 c.c. distilled water previously warmed to 60° C.

3. Mix the peptone and agar emulsions thoroughly.

4. Weigh out sodium taurocholate, 5 grammes (= 0.5

per cent.), dissolve it in 600 c.c. distilled water, and use the solution to wash the agar-peptone emulsion into a 2-litre flask.

5. Heat in the steamer at 100° C. for sixty minutes.

6. Cool to 60° C. and clarify with egg as for nutrient agar (*vide* page 150).

7. Filter through papier Chardin, using the hot-water funnel.

8. Weigh out lactose, 10 grammes (= 1 per cent.), and dissolve it in the agar.

9. If desired, add saturated aqueous solution of neutral red, 2 c.c.

10. Tube, and sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

French Proof Agar (Sabouraud).—

1. Weigh out peptone (Chassaing), 7 grammes, and emulsify it with 200 c.c. distilled water previously heated to 60° C.

2. Weigh out powdered agar, 13 grammes, and emulsify with 200 c.c. cold distilled water.

3. Mix the two emulsions and wash into a 2-litre flask with 600 c.c. distilled water.

4. Heat in the steamer for ninety minutes, to dissolve the agar.

5. Cool to 60° C. and clarify with egg as for nutrient agar (*vide* page 150).

6. Filter through papier Chardin, using the hot-water funnel.

7. Weigh out maltose, 38 grammes, and dissolve in the agar.

8. Tube, and sterilise as for nutrient agar.

English Proof Agar (Blaxall).—Substitute Witté's peptone for that of Chassaing, and proceed as for French proof agar.

Pasteur's Solution.—

1. Weigh out and mix the ash from 10 grammes of yeast; ammonium tartrate, 10 grammes; cane sugar, 100 grammes.

2. Dissolve the mixture in distilled water, 1000 c.c.

3. Tube or flask, and sterilise by the discontinuous method (three days).

Yeast Water (Pasteur).—

1. Weigh out pressed yeast, 75 grammes; place in a 2-litre flask and add 1000 c.c. distilled water.

2. Heat in the steamer at 100° C. for thirty minutes.

3. Filter through papier Chardin.

4. Tube or flask, and sterilise as for Pasteur's solution.

Cohn's Solution.—

1. Weigh out and mix

Acid potassium phosphate	5.00 grammes
Tribasic calcium phosphate	0.50 gramme
Magnesium sulphate	5.00 grammes
Ammonium tartrate	10.00 "

and dissolve in

Distilled water	1000 c.c.

2. Tube, or flask and sterilise as for Pasteur's solution.

Naegeli's Solution.—

1. Weigh out and mix

Dibasic potassium phosphate	1.0 gramme
Magnesium sulphate	0.2 "
Calcium chloride	0.1 "
Ammonium tartrate	10.0 grammes

and dissolve in

Distilled water	1000 c.c.

2. Tube or flask; sterilise as for Pasteur's solution.

Asparagin Medium (Uschinsky).—

1. Weigh out and mix

Asparagin	3.4 grammes
Ammonium lactate	10.0 "
Sodium chloride	5.0 "
Magnesium sulphate	0.2 gramme
Calcium chloride	0.1 "
Acid potassium phosphate	1.0 "

2. Dissolve the mixture in distilled water, 1000 c.c.
3. Add glycerine, 40 c.c.
4. Tube, and sterilise by the discontinuous method (three days).

Asparagin Medium (Frankel and Voges).—

1. Weigh out and mix

Asparagin	4 grammes
Sodium phosphate (neutral)	2 "
Ammonium lactate	6 "
Sodium chloride	5 "

and dissolve in

Distilled water	1000 c.c.

2. Tube, and sterilise by the discontinuous method (three days).

NOTE.—Either of the above asparagin media, after the addition of 10 per cent. gelatine or 1.5 per cent. agar, may be advantageously employed in the solid condition.

Winogradsky's Solution (for Nitric Organisms).—
1. Weigh out and mix

Potassium phosphate	1.0 gramme
Magnesium sulphate	0.5 "
Calcium chloride	0.01 "
Sodium chloride	2.00 grammes.

and dissolve in

Distilled water	1000 c.c.

2. Fill into flasks, in quantities of 20 c.c., and add to each a small quantity of freshly washed magnesium carbonate.
3. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.
4. Add to each flask containing 20 c.c. solution, 2 c.c. of a sterile 2 per cent. solution of ammonium sulphate.
5. Incubate at 37° C. for forty-eight hours and eliminate any contaminated culture flasks.

Winogradsky's Solution (for Nitrous Organisms).—
1. Weigh out and mix

Ammonium sulphate	1 gramme
Potassium sulphate	1 "

and dissolve in

Distilled water	1000 c.c.

2. Add magnesium carbonate, previously sterilised by boiling.
3. Fill into flasks and sterilise as for previous solution.

Silicate Jelly (Winogradsky).—
1. Weigh out and mix

Ammonium sulphate	0.40 gramme
Magnesium sulphate	0.05 "
Calcium chloride	0.01 "

and dissolve in

Distilled water	50 c.c.

Label—Solution A.
2. Weigh out and mix

Potassium phosphate	0.10 gramme
Sodium carbonate	0.60 "

and dissolve in

Distilled water	50 c.c.

Label—Solution B.

3. Weigh out

Silicic acid . . . . . 3.4 grammes

and dissolve in

Distilled water . . . . . 100 c.c.

4. Pour the silicic acid solution into a large porcelain basin.

5. Mix equal quantities of the solutions A and B; then add successive small quantities of the mixed salts to the silicic acid solution, stirring continuously with a glass rod, until a jelly of sufficiently firm consistence has been formed.

6. Spread a layer of this jelly over the bottom of each of several large capsules or "plates."

7. Sterilise in the steamer for twenty minutes on each of three consecutive days.

Plaster-of-Paris Discs.—

1. Take large corks, 2.5 cm. diameter, and roll a piece of stiff note-paper round each, so that about a centimeter projects as a ridge above the upper surface of the cork, and secure in position with a pin (Fig. 93).

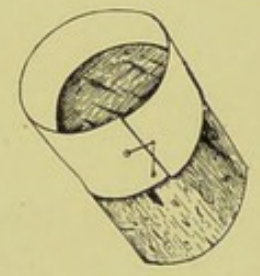


Fig. 93.—Cork and paper mould for plaster-of-Paris disc.

2. Mix plaster-of-Paris into a stiff paste with distilled water, and fill each of the cork moulds with the paste.

3. When the plaster has set, remove the paper from the corks, and lift up the plaster discs.

4. Place the plaster discs on a piece of asbestos board and sterilise by exposing in the hot-air oven to 150° C. for half an hour.

5. Remove the sterile discs from the oven by means of sterile forceps, place each inside a sterile capsule, and moisten with a little sterile water.

6. Sterilise in the steamer at 100° C. for twenty minutes on each of three consecutive days.

XI. INCUBATORS.

An incubator consists essentially of a chamber for the reception of cultivations, etc., surrounded by a water jacket, the walls of which are of metal, usually copper, and outside all an asbestos or felt jacket, or

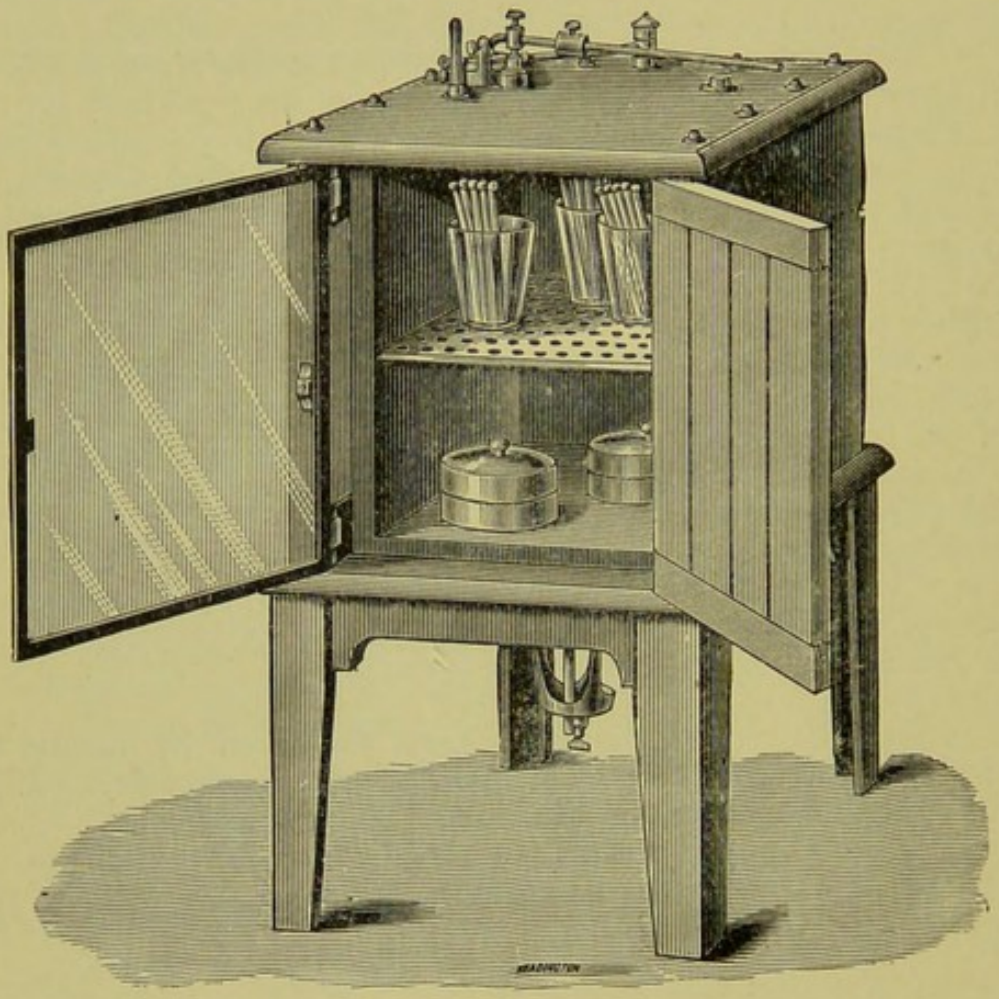


Fig. 94.—Incubator.

wooden casing. The water in the jacket is heated by gas and maintained at some constant temperature by a thermo-regulator.

Two incubators at least are required for the cultivation of bacteria in the laboratory, the one regulated

to maintain a temperature of 37° C., and known as the "hot" incubator; the other, 22° C., and known as the "cool" or "cold" incubator.

Thermo-regulators.—The thermo-regulator is the most essential portion of the incubator, as upon its efficient working depends the maintenance of a constant temperature in the cultivation chamber. It is also used in the fitting up of water and paraffin baths, and for many other purposes.

Of the many forms and varieties of thermo-regulator (other than electrical), two only are of sufficiently general use to need mention. In one of these the flow of gas to the gas-jet is controlled by the expansion or contraction of mercury within a glass bulb; in the other, by alterations in the position of the walls of a hollow metallic capsule. They are:

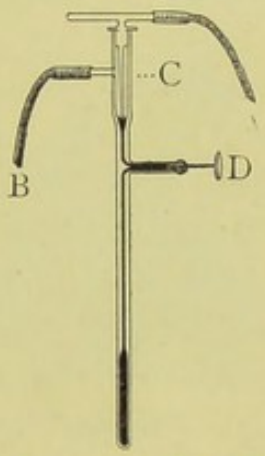


Fig. 95.—Reichert's thermo-regulator.

(*a*) *Reichert's* (Fig. 95), which consists of a bulb containing mercury. Gas enters at A, and passes out to the jet by B. As the temperature rises the mercury expands and cuts off the main gas supply, thereby reducing the temperature. As the temperature falls the mercury contracts and reopens the narrow tube C. By means of a thumbscrew D (which mechanically raises or lowers the column of mercury irrespective of the temperature) and the aid of a thermometer the apparatus can be set to keep the incubator at any desired temperature. With this form a special gas burner is required, with separate supply of gas to a pilot jet at the side.

(*b*) *Capsule regulator* consists of a metal capsule filled with a liquid which boils at the required temperature, and hermetically sealed; this is adjusted in the interior of the incubator. Soldered to the upper side of the

capsule is a thick piece of metal having a central depression which receives the lower end of a rigid rod, by means of which the movements of the walls of the capsule are transmitted to the gas valve fixed outside the incubator.

Figure 96 represents a well-known form of capsule regulator. A is the inlet for gas, C the outlet to burner heating the water jacket, B D a lever pivoted to standards at G, and acted upon by the capsule, through the needle which enters the socket below the screw P.

The construction of the valve is such that, whenever the end B of the lever B D presses on the disc below the end B, the main supply of gas is entirely cut off. At such times, however, a very small portion of gas passes from A to C, through an aperture inside the valve, the size of which aperture can be adjusted by the screw needle S, hence the gas flame below the incubator is never extinguished.

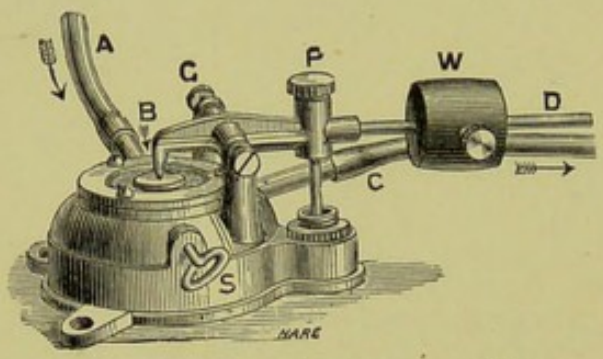


Fig. 96.—Capsule thermo-regulator.

The expansion of the metal walls of the capsule, owing to the boiling of its contents, provides the motive force for acting upon the lever B D, and as this expansion only takes place at a predetermined temperature, the lever will only be acted upon when the critical temperature is reached, no sensible effect being produced at even 1° C. below that at which the capsule is desired to act.

W is a weight sliding on the lever rod D, by means of which the boiling-point of the liquid in the capsule can be slightly retarded, and a range of about two degrees obtained with any particular capsule.

XII. METHODS OF CULTIVATION.

CULTIVATIONS of micro-organisms are usually prepared in the laboratory in one of three ways:

Tube cultures.
Plate cultures.
Hanging-drop cultures.

These may be incubated either **aerobically** (*i. e.*, in the presence of oxygen) or **anaerobically** (*i. e.*, in the absence of oxygen, or in the presence of an indifferent gas, such as hydrogen, nitrogen, or carbon dioxide).

With regard to the temperature at which the cultivations are grown, it may be stated as a general rule that all media rendered solid by the addition of gelatine are incubated at 20° C., or at any rate at a temperature not exceeding 22° C. (that is, in the "cold" incubator); whilst fluid media and all other solid media are incubated at 37° C. (that is, in the "hot" incubator). Exceptions to this rule are numerous. For instance, in studying the growth of the psychrophylic bacteria, the yeasts and the moulds, the cold incubator is employed for all media.

Tube cultivations are usually packed in the incubator in small tin cylinders, such as those in which American cigarettes are sold. Beakers or tumblers may be used for the same purpose, but are not so convenient.

AEROBIC.

The Preparation of Tube Cultivations.

The preparation of a tube cultivation consists in:

(*a*) Inoculating a tube of sterile nutrient medium with a portion of the material to be examined.

(*b*) Incubating the inoculated tube at a suitable temperature.

The details of the first of these processes must be varied somewhat according to whether the tubes of nutrient media are inoculated or "planted" from—

1. Pre-existing cultivations.
2. Previously collected morbid material.
3. The animal body direct.

The method of preparing tube cultivations from pre-existing cultivations is as follows:

1. Fluid Media (*e. g.*, Nutrient Bouillon).—

1. Flame the cotton-wool plug of the tube containing the cultivation and also that of the tube of sterile bouillon.

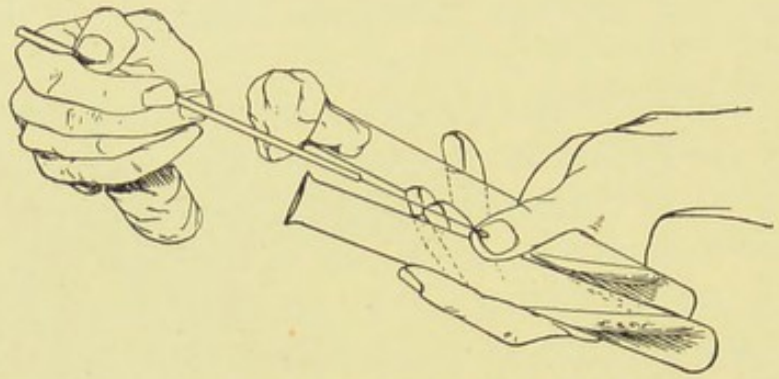


Fig. 97.—Inoculating tubes, seen from the front.

2. Hold the two tubes, side by side, between the left thumb and the first and third fingers, allowing the sealed ends to rest on the dorsum of the hand, and separating the mouths of the tubes (which are pointed to the right) by the tip of the second finger. Keep the tubes as nearly horizontal as is possible without allowing the fluid in the bouillon tube to reach the cotton-wool plug.

3. Sterilise the platinum loop and allow it to cool.[1]

4. Grasp the plug of the tube containing the cultivation between the little finger and palm of the hand and remove it from the tube.

[1] See also method of opening and closing culture tubes, page 69.

5. Grasp the plug of the bouillon tube between the fourth finger and the ball of the thumb and remove it from the tube.

6. Pass the platinum loop into the tube containing the culture,—do not allow the loop to touch the sides of the tube, or the handle to touch the medium,—and remove a small portion of the growth; withdraw the loop from the tube, keeping the infected side of the loop downwards.

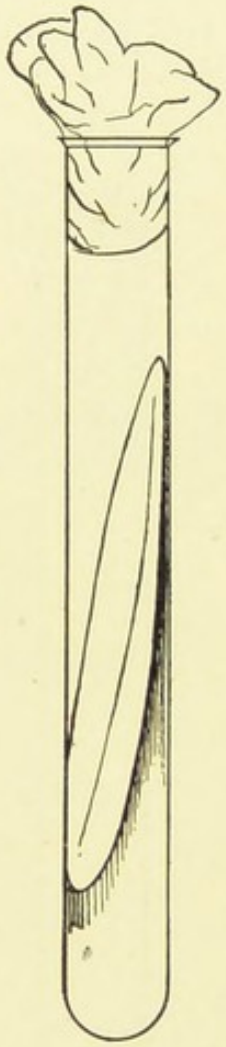


Fig. 98.—Streak tube.

7. Pass the loop into the bouillon tube almost down to the level of the fluid, reverse the loop so that the infected side faces upwards, emulsify the portion of the growth in the moisture adhering to the side of the tube which is uppermost. Withdraw the loop.

8. Replug both tubes.

9. Sterilise the platinum loop.

10. Label the bouillon tube with (*a*) the name of the organism and (*b*) the date of inoculation.

11. Incubate.

2. Solid Media.—Solid media are stored in tubes in one of two ways:

1. Oblique tube or slanted tube (Fig. 98), in which the medium has been allowed to solidify whilst the tube was retained in an inclined position, so forming an extensive surface of medium extending from the bottom of the tube almost to its mouth.

This is employed for "streak" or "smear" cultivations (*Strichcultur*).

NOTE.—Gelatine and agar oblique tubes should be freshly "slanted" before use.

2. Straight tube (Fig. 99), in which the medium forms a cylindrical mass in the lower portion of the tube and presents an upper surface which is at right angles to the long axis of the tube.

This is employed for "stab" or "stick" cultivations (*Stichcultur*), or by inoculating the medium whilst fluid, and allowing to solidify in this position, for "shake" cultivations.

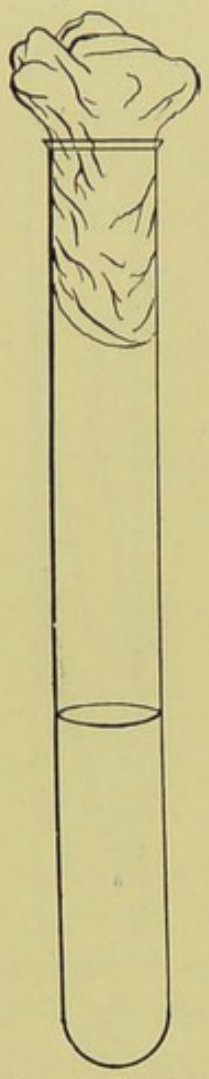


Fig. 99.—Straight tube.

Streak Culture.—

1. Flame the plugs, sterilise the platinum loop (or spatula). Open the tubes and charge the loop as in previous inoculation.

2. Pass the infected loop to the bottom of the tube to be inoculated and draw it, as lightly as possible, along the centre of the surface of the medium, terminating the "streak" over the thin layer of medium near the mouth of the tube.

3. Replug the tubes, sterilise the platinum loop.

4. Label the newly inoculated tube and incubate.

Smear Culture.—Proceed generally as in streak culture, but rub the infected loop all over the surface of the medium, instead of restricting the inoculation to a narrow line.

Stab Culture.—

1. Flame the plugs, open the tubes, sterilise the platinum needle and charge it with the inoculum as in the previous cultivations.

2. Pass the platinum needle into the tube to be inoculated until it touches the centre of the surface of the medium. Now thrust it deeply into the substance of the medium, keeping the needle as nearly as

possible in the axis of the cylinder of medium. Then withdraw the needle.

3. Replug the tubes. Sterilise the platinum needle.

4. Label the newly planted tube and incubate.

Shake Culture.—

1. Liquefy a tube of nutrient gelatine (or agar, or other similar medium), by heating in a water-bath (Fig. 100).

2. Inoculate the liquefied medium and label it, etc., precisely as if dealing with a tube of bouillon.

3. Place the newly planted tube in the upright position (*e. g.*, in a test-tube rack) and allow it to solidify.

4. Label the tube; when solid, incubate.

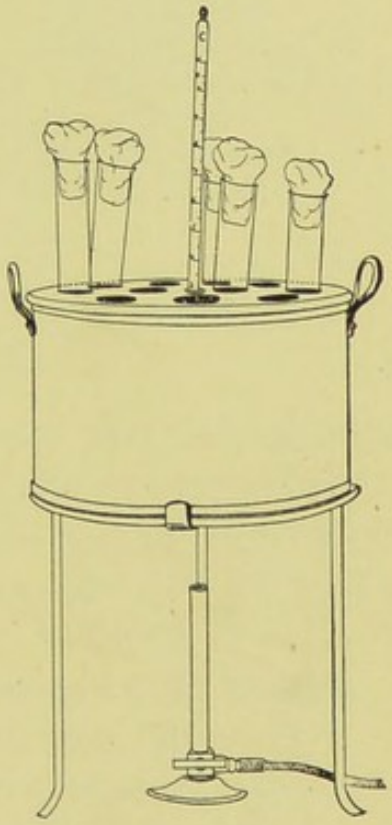


Fig. 100.—Handy form of water-bath for melting tubes of agar and gelatine previous to slanting them; or to making shake cultures.

The Preparation of Plate Cultures.

If a small number of bacteria are suspended in liquefied gelatine, agar, or other similar medium, and the infected medium spread out in an even layer over a flat surface and allowed to solidify, each individual micro-organism becomes fixed to a certain spot and its further development is restricted to the vicinity of this spot. After a variable interval the growth of this organism becomes visible to the naked eye as a "colony." This is the principle upon which the method of plate cultivation is based. The method itself is as follows:

Apparatus Required.—

1. Tripod levelling stand.
2. Large shallow glass dish, with a square sheet of plate glass to cover it.
3. Spirit level.
4. Case of sterile Petri dishes.
5. Tubes of sterile nutrient media, gelatine (or agar) previously liquefied by heating in the water-bath and cooled to 42° C., otherwise the heat of the medium would destroy many, if not all, of the bacteria introduced.
6. Tube of cultivation to be planted from.
7. Platinum loop.
8. Bunsen burner.
9. Grease pencil.

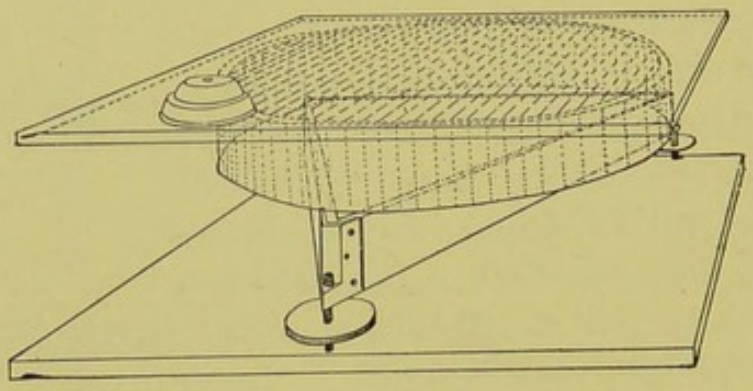


Fig. 101.—Plate-levelling stand.

Method of "Pouring" Plates.—

1. Place the glass dish on the levelling tripod (Figs. 101, 102), fill it with ice water if gelatine plates are to be poured, or with water at 50° C. if agar is to be used; cover it with the square sheet of glass.

2. Place the spirit level on the sheet of glass and by means of the levelling screws adjust the surface of the glass to the horizontal.

3. Place three sterile Petri dishes in a row on the surface of the glass plate and number them 1, 2, and 3, from left to right.

4. Number the previously liquefied tubes of nutrient media 1, 2, and 3. Flame the plugs and see that each plug can be readily removed from the mouth of its tube.

5. Add one loopful of the inoculum to tube No. 1, treating the liquefied medium as bouillon. After replugging, grasp the tube near its mouth by the thumb and first finger of the right hand, and with an even circular movement of the whole arm, diffuse the inocu-

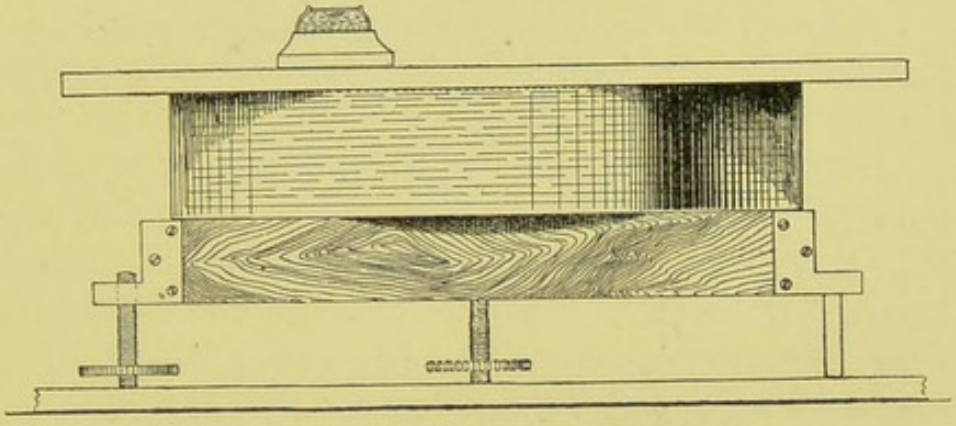


Fig. 102.—Plate-levelling stand, side view.

lum throughout the medium; avoid jerky movements, as these cause bubbles of air to form in the medium.

6. Sterilise the platinum loop, and add two loopfuls of diluted inoculum to tube No. 2, and mix as before.

7. In a similar manner transfer three loopfuls of

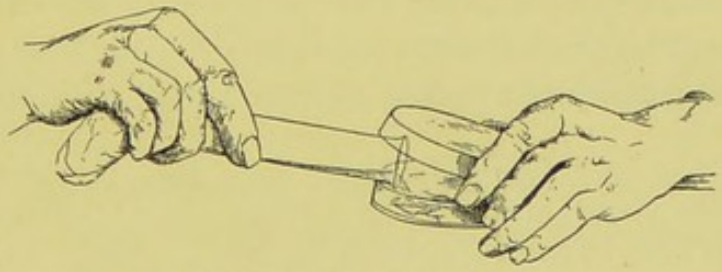


Fig. 103.—Pouring plates.

liquefied medium from tube No. 2 to tube No. 3, and mix thoroughly.

8. Flame the plug of tube No. 1, remove it, then flame the lips of the tube; slightly raise the cover of Petri dish No. 1, introduce the mouth of the tube; then, elevating the bottom of the tube, pour the liquefied medium into the Petri dish, to form a thin layer.

Remove the mouth of the tube and close the "plate." If the medium has failed to flow evenly over the bottom of the plate, raise the plate from the levelling platform and by tilting in different directions rectify the fault.

9. Pour plates No. 2 and No. 3, in a similar manner, from tubes Nos. 2 and 3.

10. Label the plates with the distinctive name or number of the inoculum, the number of the dilution, also the date.

11. Place in the cool incubator for three or more days, as may be necessary.

In this way colonies may be obtained quite pure and separate from each other.

In plate No. 1, probably, the colonies will be so numerous and crowded, and therefore so small, as to render it useless. In plate No. 2 they will be more widely separated, but usually No. 3 is the plate reserved for careful examination, as in this the colonies are usually widely separated, few in number, and large in size.

Agar plates are poured in a similar manner, but the agar must be melted in boiling water and then allowed to cool to 45° C. or 42° C. in a carefully regulated water-bath before being inoculated, and the entire process must be carried out very rapidly, otherwise the agar will have solidified before the operation is completed.

NOTE.—In pouring plates, tube No. 1 (for the first dilution) very rarely gives a plate that is of any practical value; consequently it is frequently replaced by a tube of bouillon or sterile salt solution, and plate No. 1 is not poured.

Hanging-drop Cultivation.

(a) **Fluid Media.**—

1. Prepare first and second dilutions of the inoculum as directed for plate cultivations (*vide* page 182, sections 4 to 6), substituting tubes of nutrient broth for the liquefied gelatine.

2. Sterilise a hanging-drop slide by washing thoroughly in water and drying, then plunging it into a beaker of absolute alcohol, draining off the greater part of the spirit, grasping the slide in a pair of forceps, and burning off the remainder of the alcohol in the flame.

3. Place the hanging-drop slide on a piece of blotting paper moistened with 2 per cent. lysol solution and cover it with a small bell glass that has been rinsed out with the same solution and *not dried.*

4. Raise the bell glass slightly and smear sterile vaseline around the rim of the metal cell by means of a sterile spatula of stout platinum wire.

5. Remove a clean cover-slip from the alcohol pot with sterile forceps and burn off the alcohol; again raise the bell glass and place the sterile cover-slip on the blotting paper by the side of the hanging-drop slide.

6. Remove a drop of the broth from the second dilution tube with a large platinum loop; raise the bell glass and deposit the drop on the centre of the cover-slip. Sterilise the loop.

7. Raise the bell glass sufficiently to allow of the cover-slip being grasped with forceps, inverted, and adjusted over the cell of the hanging-drop slide. Remove the bell glass altogether and press the cover-slip firmly on to the cell.

8. Either incubate and examine at definite intervals, or observe continuously with the microscope, using a warm stage if necessary (Fig. 40).

(b) **Solid Media.**—Observing precisely similar technique, a few drops of liquefied gelatine or agar from the second dilution tube may be run over the surface of the sterile cover-slip and a hanging-drop plate cultivation thereby prepared.

This method is extremely useful in connection with the study of yeasts, and in this connection the circular cell on the hanging-drop slide is replaced by a rectangu-

lar cell some 38 by 19 mm., and the gelatine spread over a cover-slip of similar size. After sealing down the preparation, the upper surface of the cover-slip may be ruled into squares by the aid of the grease pencil or a writing diamond.

ANAEROBIC CULTIVATIONS.

Numerous methods have been devised for the cultivation of anaerobic bacteria, the majority requiring the employment of special apparatus. The principle upon which any method is based and upon which it depends for its success falls under one or another of the following headings:

(a) **Exclusion of air** from the cultivation.

(b) **Exhaustion of air** from the vessel containing the cultivation by means of an air pump—*i. e.*, cultivation *in vacuo*.

(c) **Absorption of oxygen** from the air in contact with the cultivation by means of pyrogallic acid rendered alkaline with caustic soda—*i. e.*, cultivation in an atmosphere of nitrogen.

(d) **Displacement of air** by an indifferent gas, such as hydrogen or coal gas—*i. e.*, cultivation in an atmosphere of hydrogen.

(e) A combination of two or more of the above methods.

A selection of the simplest and most generally useful methods is given here.

Whenever possible, the nutrient media that are employed in any of the processes should contain some easily oxidisable substance, such as sodium formate (0.4 per cent.) or sodium sulphindigotate (0.1 per cent.), which will absorb all the available oxygen held in solution by the medium. The further addition of glucose, 2 per cent., favors the growth of anaerobic bacteria (*vide* glucose formate bouillon, page 142; sulphindigotate bouillon, page 143, etc.).

Further, it is advisable to seal all joints between india-rubber stoppers and tubulures or the mouths of the tube with melted paraffin.

(A) **Method I** (Hesse's Method).—

1. Make a stab culture in gelatine or agar, choosing for the purpose a straight tube containing a deep column of medium, and thrusting the inoculating needle to the bottom of the tube.

2. Pour a layer of sterilised oil (olive oil, vaseline, or petroleum), 1 or 2 cm. deep, upon the surface of the medium.

3. Incubate.

Method II.—

1. Make an Esmarch's roll cultivation in the usual way.

2. Fill the lumen of the tube with sterile gelatine that has been liquefied by heat and cooled in the water-bath to 15° C. (At this temperature the gelatine will remain fluid for only a few minutes.)

3. Incubate.

NOTE.—This method is but rarely employed.

Method III.—This method is only available when dealing with pure cultivations.

1. Liquefy a tube of gelatine (or agar) by heat, pour it into a Petri dish, and allow it to solidify.

2. Inoculate the surface of the medium in one spot only.

3. Remove a cover-slip from the pot of absolute alcohol with sterile forceps; burn off the alcohol in the gas flame.

4. Lower the now sterile cover-slip carefully on to the inoculated surface of the medium, carefully excluding air bubbles, and press it down firmly with the points of the forceps. (A sterile disc of mica may be substituted for the cover-slip.)

5. Incubate.

Method IV (Roux's Physical Method).—

1. Prepare tube cultures of fluid media (or solid media rendered fluid by heat) in the usual way.

2. Aspirate some of the inoculated media into capillary pipettes.

3. Seal both ends of each pipette in the blowpipe flame.

4. Incubate.

Method V (Roux's Biological Method).—

1. Plant a deep stab, as in method I.

2. Pour a layer, 1 or 2 cm. deep, of broth cultivation of an aerobe—*e. g.*, B. aquatilis sulcatus or B. prodigiosus—upon the surface of the medium; or an equal depth of liquefied gelatine, which is then inoculated with the aerobic organism.

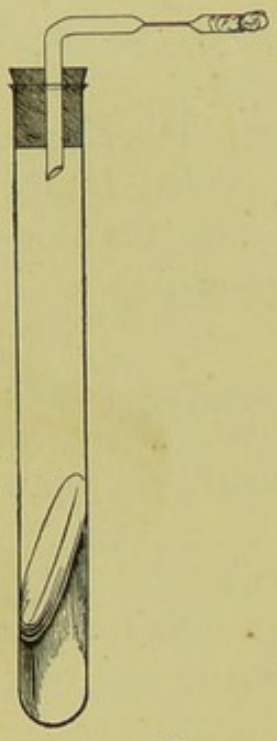


Fig. 104.—Vacuum culture.

3. Incubate.

The growth of the aerobe will use up all the oxygen that reaches it and will not allow any to pass through to the medium below, which will consequently remain in an anaerobic condition.

(B) **Method VI.**—

1. Prepare tube or flask cultivations in the usual way.

2. Replace the cotton-wool plug by an india-rubber stopper perforated with one hole and fitted with a length of glass tubing which has a constriction about 3 cm. above the stopper and is then bent at right angles (Fig. 104). The stopper and glass tubing are sterilised by being boiled in a beaker of water for five minutes.

3. Connect the tube leading from the culture vessel with a water or air pump, interposing a Wulff's bottle fitted as a wash-bottle and containing sulphuric acid.

4. Exhaust the air from the culture vessel.

5. Before disconnecting the apparatus, seal the glass tube from the culture vessel at the constriction, using the blowpipe flame.

6. Incubate.

(C) **Method VII** (Buchner's Method).—

Apparatus and Solutions Required.—

Buchner's tube (a stout glass test-tube 23 cm. long and 4 cm. in diameter, fitted with india-rubber stopper, Fig. 105).
Ten per cent. aqueous solution of pyrogallic acid.[1]
Dekanormal solution of caustic soda.

METHOD.—

1. Prepare the tube cultivation in the usual way.

2. Moisten the india-rubber stopper of the Buchner's tube with water and see that it fits the mouth of the tube accurately.

3. Remove the stoppers from the pyrogallic acid and caustic soda bottles.

4. Run about 10 c.c. of the pyrogallic solution into the Buchner's tube (roughly, use 5 c.c. pyrogallic solution for every 100 c.c. air capacity of the receiving vessel).

5. Add about 1 c.c. of the soda solution.

6. Place the inoculated tube inside the Buchner's tube.

7. Fit the india-rubber stopper tightly into the mouth of the Buchner's tube.

NOTE.—*Sections 4 to 7 must be performed as quickly as possible.*

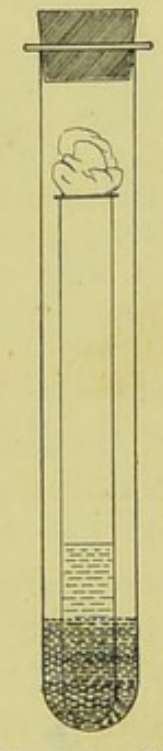


Fig. 105.—Buchner's tube.

8. Restopper the pyrogallic acid and caustic soda bottles.

9. Place Buchner's tube in a wire support, and incubate.

[1] One and a half cubic centimetres of hydrochloric acid should be added to every 1000 c.c. of the stock pyrogallic solution to prevent oxidation.

Method VIII (Wright's Method).—

1. Prepare tube cultivation in the usual way.

2. Cut off that portion of the cotton-wool plug projecting above the mouth of the tube with scissors, then push the plug into the tube for a distance of 2 or 3 cm.

3. By means of a pipette drop about 1 c.c. of the pyrogallic acid solution on to the plug. It will immediately be absorbed by the cotton-wool.

4. With another pipette run in an equal quantity of the caustic soda solution.

5. Quickly close the mouth of the tube with a tightly fitting india-rubber stopper.

6. Incubate.

(D) **Method IX.**—

Apparatus Required.—

Small Ruffer's or Woodhead's flask (Fig. 23).
Sterile india-rubber stopper.
India-rubber tubing.
Glass tubing.
Metal screw clips.
Cylinder of compressed hydrogen or Kipp's hydrogen apparatus.

METHOD.—

1. Sterilise a glass vessel, shaped as in a Ruffer's or Woodhead's flask, in the hot-air oven. (The tubulure and the side tubes are plugged with cotton-wool.) After sterilisation, fix a short piece of rubber tubing occluded by a metal clip to each side tube.

2. Inoculate a large quantity (*e. g.*, 200 c.c.) of the medium. Where solid media are employed they must first be liquefied by heat.

3. Remove the cotton-wool plug from the tubulure and pour the inoculated medium into the glass vessel.

4. Close the tubulure by means of an india-rubber stopper previously sterilised by boiling in a beaker of water.

5. Connect up the india-rubber tubing on one of the side tubes with a cylinder of compressed hydrogen (or the delivery tube of a Kipp's hydrogen apparatus, Fig. 106), interposing a short piece of glass tubing; and in like manner connect a long piece of rubber tubing which should be led into a basin of water, to the opposite side tube.

6. Open both metal clips and pass hydrogen through the vessel until the atmospheric air is replaced by

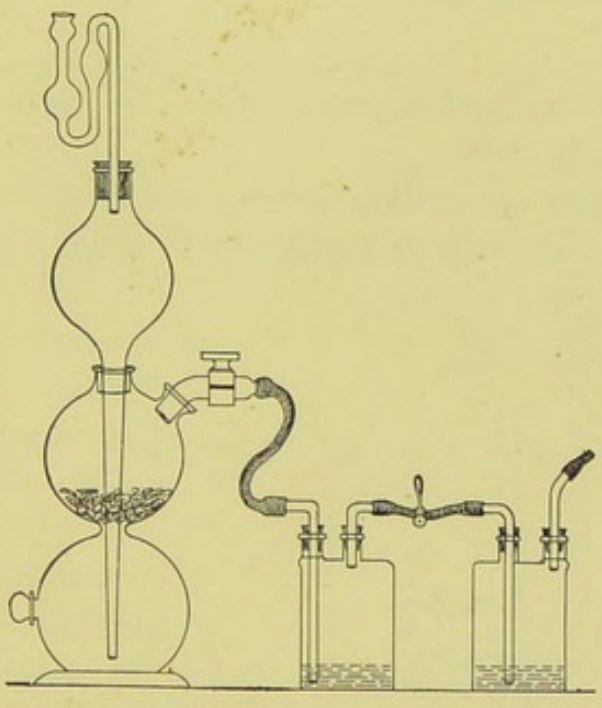


Fig. 106.—Kipp's hydrogen apparatus with two washing bottles containing lead nitrate and silver nitrate solutions respectively to remove impurities.

hydrogen. This is determined by collecting some of the gas which bubbles through the water in the basin in a test-tube and testing it by means of a lighted taper.

7. Close the metal clip on the tube through which the gas is entering; close the clip on the exit tube.

8. Disconnect the gas apparatus.

9. Incubate.

Method X (Botkin's Method).—

Apparatus Required.—

Large glass dish 20 cm. diameter and 8 cm. deep. Flat leaden cross slightly shorter than the internal diameter of the glass dish.
Bell glass about 15 cm. diameter and 20 to 25 cm. high.
Metal frame for plate cultivations.
Or, glass battery jar for tube cultivations.
Cylinder of compressed hydrogen.
Rubber tubing.
Two pieces of U-shaped glass tubing (each arm 8 cm. in length).
Half a litre of glycerine.

METHOD.—

1. Place the leaden cross inside the glass dish, resting on the bottom.

2. Prepare the cultivations in the usual way.

3. Place the tube cultivations in a glass battery jar (or the plate cultivations on a metal frame), resting on the centre of the leaden cross.

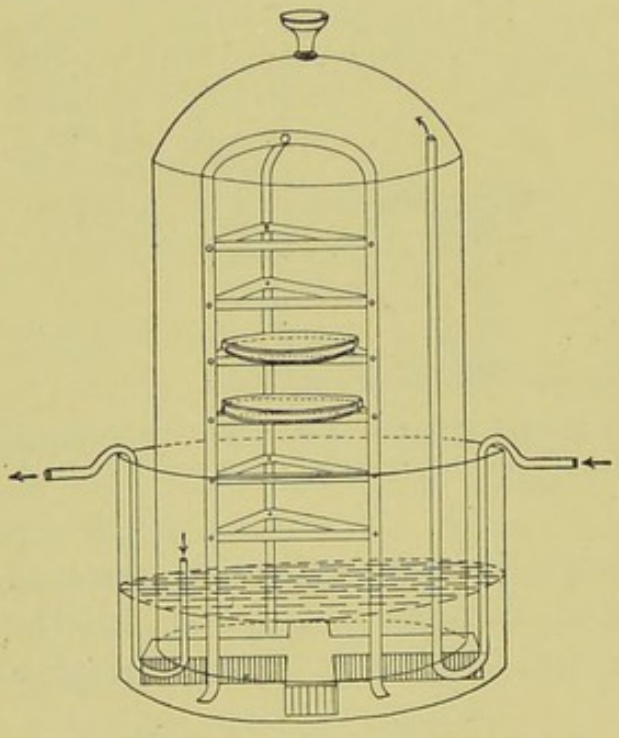


Fig. 107.—Botkin's apparatus.

4. Cover the cultivations with the bell jar.

5. Adjust the U-shaped pieces of glass tubing in a vertical position on opposite side of the bell jar, one arm of each inside the jar, the other outside. Fix a short length of rubber tubing clamped with a metal clip to each of the outside arms.

6. Fill the glass dish with glycerine to a depth of about 5 cm. (Fig. 107).

7. Connect up one U-shaped tube with the hydrogen cylinder by means of rubber tubing. Replace the atmospheric air by hydrogen, as in method IX.

8. Clamp the tubes and disconnect the gas apparatus.

9. Incubate.

Method XI (Novy's Method).—

Apparatus Required.—

Jar for plate cultivations (Fig. 108).
Or, jar for tube cultivations (Fig. 109).
Lubricant for stopper of jar (beeswax 1 part, olive oil 4 parts).
Rubber tubing.
Cylinder of compressed hydrogen.

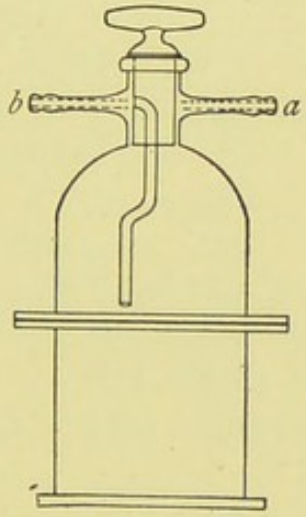


Fig. 108.—Novy jar for plate cultivations.

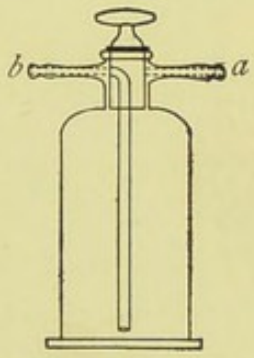


Fig. 109.—Novy's jar for tube cultivations.

METHOD.—

1. Prepare cultivations in the usual way.

2. Place these inside the jar.

3. Lubricate the stopper, and insert it in the mouth of the jar, with the handle in a line with the two side tubes.

4. Connect up the delivery tube *a* with the hydrogen gas supply by means of rubber tubing.

5. Attach a piece of rubber tubing to the exit tube *b* and collect samples of the issuing gas (over water) and test from time to time.

6. When the air is completely displaced by hydrogen, turn the handle of the stopper at right angles to the line of the entry and exit tubes; this seals the orifice of both tubes.

7. Disconnect the gas apparatus and incubate.

(E) **Method XII** (Bullock's Method).—

Apparatus Required.—

Bullock's jar.
Pot of resin ointment.
Small glass dish 14 cm. diameter by 5 cm. deep.
Vessel for tube cultures or metal rack for plate cultures.
Pyrogallic acid powder.
Cylinder of compressed hydrogen.
Geryk or other air pump.
Rubber tubing.
Glass tubing.
Small beaker of dekanormal caustic soda.
Small beaker of water.

METHOD.—

1. Prepare the cultivations in the usual way.

2. Place the glass dish in the centre of the glass slab, and stand the cultivations inside this.

3. Place a quantity of dry pyrogallic acid in a heap at one side of the glass dish.

4. Smear the flange of the bell jar with resin ointment and apply the jar firmly to the glass slab, covering the cultivations,—so arranged that the long tube passes with its lower end into the glass dish at a point directly opposite to the pyrogallic acid powder. (This is to prevent the tube getting blocked with pyrogallic acid during the next step.) Lubricate the two stop-cocks with resin ointment.

5. Connect up the short tube *a* with the gas-supply by means of rubber tubing and open both stop-cocks.

6. When the air is displaced, shut off the stop-cock of the entry tube, then that of the exit tube *b*.

7. Connect a long, straight piece of glass tubing to the long tube *b* by means of a piece of rubber tubing;

and connect up the short tube *a* to the air pump by means of pressure tubing.

8. Open the stop-cock of tube *a* and aspirate a small quantity, say 100 c.c., of gas by means of the air pump, so creating a slight vacuum. Then shut off the stop-cock and disconnect the air pump.

9. Dip the long glass tube (connected with *b*) into the beaker of soda solution; open the stop-cock and the alkali will run down the long tube and come into contact with the dry pyrogallic acid.

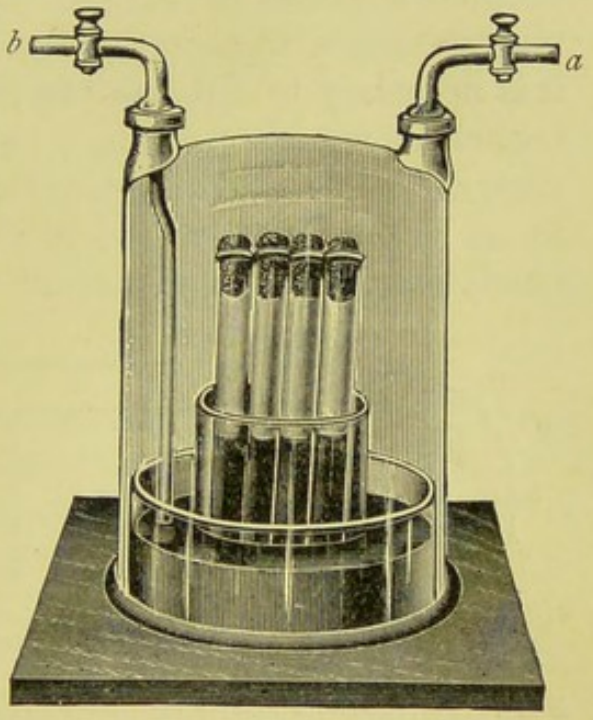


Fig. 110.—Bullock's jar.

10. When 2 or 3 c.c. of soda solution have been run in, shut off the stop-cock, remove the glass tube from the soda solution, and place it in the beaker of water.

11. In a similar manner run in a few cubic centimetres of water and again shut the stop-cock. (This serves to wash out the tubes and prevents the alkali collecting at the stop-cock and exerting a corrosive action on the glass.)

12. Incubate.

This last method is the most satisfactory for anaerobic cultivations, as by its means complete anaerobiosis can be obtained with the least expenditure of time and trouble.

XIII. METHODS OF ISOLATION.

THE work in the preceding sections, arranged to demonstrate the chief biological characters of bacteria in general, is intended to be carried out by means of pure cultivations of various organisms. But before undertaking a systematic study of selected bacteria, it is necessary to indicate the chief methods by which one or more organisms may be isolated in a state of purity from a mixture; whether that mixture exists as an impure cultivation, in pus and other morbid exudations, infected tissues, or water or food-stuffs.

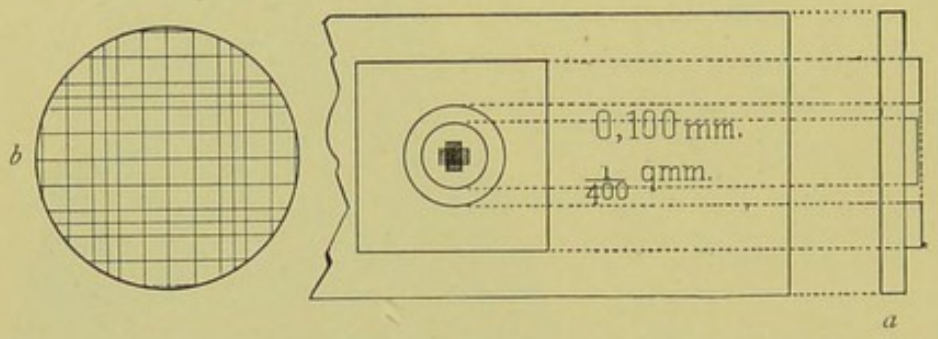


Fig. 111.—Hæmatocytometer cell, showing, *a*, section through the centre of the cell, and *b*, a magnified image of the cell rulings.

Before the introduction of solid media the only method of obtaining pure cultivations was by "dilution"—by no means a reliable method. "Dilution" consisted in estimating approximately the number of bacteria present in a given volume of fluid (by means of a graduated-celled slide resembling a hæmatocytometer, Fig. 111), diluting the fluid by the addition of sterile water or bouillon until a given volume (usually 1 c.c.) of the dilution should contain but one organism. By planting this volume of the fluid into several tubes or flasks of nutrient media, it occasion-

ally happened that the resulting growth was the product of one individual microbe. A method so uncertain is now fortunately replaced by many others, both reliable and convenient, and in those methods selected for description here, the isolation of the required bacteria is effected—

1. By plate cultivation:
(*a*) Gelatine.
(*b*) Agar.
(*c*) Serum agar.
(*d*) Blood agar.
2. By Esmarch's roll cultivation:
(*a*) Gelatine.
(*b*) Agar.
3. By serial cultivation.
4. By differential media.
(*a*) Selective.
(*b*) Deterrent.
5. By differential incubation.
6. By differential sterilisation.
7. By differential atmosphere cultivation.
8. By animal inoculation.

The selection of the method to be employed in any specific instance will depend upon a variety of circumstances, and often a combination of two or more will ensure a quicker and more reliable result than a rigid adherence to any one method. Experience is the only reliable guide, but as a general rule the use of either the first or the third method will be found most convenient, affording as they do an opportunity for the simultaneous isolation of more than one of the bacteria present in a mixture.

1. Plate Cultivations.—

(*a*) *Gelatine* (*vide* page 145).
(*b*) *Agar* (*vide* page 149).
(*c*) *Alkaline serum agar* (*vide* page 157).

These plates are poured in a manner precisely

similar to that adopted for nutrient gelatine and nutrient agar plates (*vide* page 182).

(*c'*) *Serum Agar.*—

1. Melt three tubes of nutrient agar, label them 1, 2, and 3, and place them, with three tubes of sterile fluid serum, also labelled 1, 2, and 3, in a water-bath regulated at 45° C.; allow sufficient time to elapse for the temperature of the contents of each tube to reach that of the water-bath.

2. Make three dilutions of the inoculum in the three liquid serum tubes, treating them exactly as if they were tubes of liquefied gelatine (*vide* page 183); replace them in the water-bath.

3. Take serum tube No. 1 and agar tube No. 1. Flame the plugs and remove them from the tubes (retaining the plug of the agar tube in the hand); flame the mouths of the tubes, pour the serum into the tube of liquefied agar and replace the plug of the agar tube.

4. Mix thoroughly and pour plate No. 1 *secundum artem.*

5. Treat the remaining dilutions in a similar fashion, and pour plates Nos. 2 and 3 in the usual way.

6. Label and incubate.

(*d*) *Human Blood Agar.*—

1. Melt a tube of sterile agar and pour it into a sterile plate; let it set.

2. Collect a few drops of human blood, under all aseptic conditions, in a sterile capillary pipette.

3. Raise the cover of the Petri dish very slightly, insert the extremity of the capillary pipette, and deposit the blood on the centre of the agar surface. Close the dish.

4. Charge a platinum loop (or a sterilised camel's hair brush) with a small quantity of the inoculum. Raise the cover of the plate, introduce the loop, mix

its contents with the drop of blood, and finally smear the mixture over the surface of the agar.

5. Withdraw the loop and close the plate.

6. Label and incubate.

(If considered necessary, two, three, or more similar plates may be inoculated in series.)

2. Esmarch's Roll Cultivation.—

(*a*) *Gelatine.*—

1. Liquefy three tubes of gelatine by heat.

2. Prepare three dilutions of the inoculum (as described for plate cultivations).

3. Roll the tubes, held almost horizontally, in a groove made in a block of ice, until the gelatine has

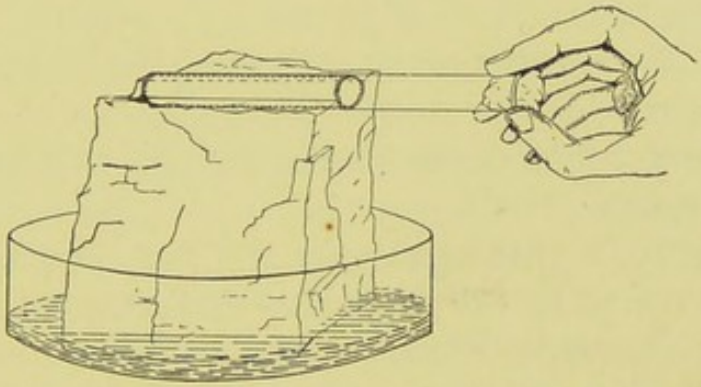


Fig. 112.—Esmarch's roll culture on block of ice.

set in a thin film on the inner surface of tube (Fig. 112); or under the cold-water tap.

(*b*) *Agar* roll cultures are made in precisely the same way as gelatine roll cultures, but in order that the medium may adhere firmly to the glass, the agar used for the purpose should have 1 per cent. gelatine or 1 per cent. gum arabic added to it before sterilisation.

Roll cultivations, which served a most important purpose in the days before the introduction of Petri dishes for plate cultivations, are now seldom prepared, and are in point of fact practically obsolete.

3. Serial Cultivations.—These are usually made

upon agar or blood-serum, although gelatine may also be used. The method is as follows:

1. Take at least six "slanted" tubes of media and number them consecutively.

2. Flame all the plugs and see that each can be readily removed.

3. Charge the platinum loop with a small quantity of the inoculum, observing the usual routine, and plant tube No. 1, smearing thoroughly all over the surface. If any water of condensation has collected at the bottom of the tube, use this as a diluent before smearing the contents of the loop over the surface of the medium.

4. Without sterilising or recharging the loop, inoculate tube No. 2.

5. In like manner plant the remainder of the tubes in the series.

6. Label with distinctive name or number, and date; incubate.

The growth that ensues in the first two or three tubes of the series will probably be so crowded as to be useless. Towards the end of the series, however, discrete colonies will be found, each of which can be transferred to a fresh tube of nutrient medium without risk of contamination from the neighboring colonies.

4. Differential Media.—

(*a*) *Selective.*—Some varieties of media are specially suitable for certain species of bacteria and enable them to overgrow and finally choke out other varieties; *e. g.*, wort is the most suitable medium-base for the growth of torulæ and yeasts and must always be employed when pouring plates for the isolation of these organisms. To obtain a pure cultivation of yeast from a mixture containing bacteria as well, it is sufficient to inoculate wort from the mixture and incubate at 37° C. for twenty-four hours. Plant a fresh tube of wort from the resulting growth and incubate. Repeat the pro-

cess once more, and from the growth in this third tube plant a streak on wort gelatine, and incubate at 20° C. The resulting growth will almost certainly be a pure culture of the yeast.

(*b*) *Deterrent.*—The converse of the above also obtains. Certain media possess the power of inhibiting the growth of a greater or less number of species. For instance, media containing carbolic acid to the amount of 1 per cent. will inhibit the growth of practically everything but the Bacillus coli communis.

5. Differential Incubation.—In isolating certain bacteria, advantage is taken of the fact that different species vary in their optimum temperature. A mixture containing the Bacillus typhosus and the Bacillus aquatilis sulcatus, for example, may be planted on two slanted agar tubes, the one incubated at 40° C., and the other at 12° C. After twenty-four hours' incubation the first will show a pure cultivation of the Bacillus typhosus, whilst the second will be an almost pure culture of the Bacillus aquatilis.

6. Differential Sterilisation.—

(*a*) *Non-sporing Bacteria.*—Similarly, advantage may be taken of the varying thermal death-points of bacteria. From a mixture of two organisms whose thermal death-points differ by, say, 4° C.—*e. g.*, Bacillus pyocyaneus, thermal death-point 55° C., and Bacillus mesentericus vulgatus, thermal death-point 60° C.—a pure cultivation of the latter may be obtained by heating the mixture in a water-bath to 58° C. and keeping it at that point for ten minutes. The mixture is then planted on to fresh media and incubated, when the resulting growth will be found to consist entirely of the B. mesentericus.

(*b*) *Sporing Bacteria.*—This method is found to be of even greater practical value when applied to the differentiation of a spore-bearing organism from one which does not form spores. In this case the mixture

is heated in a water-bath at 80° C. for fifteen to twenty minutes. At the end of this time the non-sporing bacteria are dead, and cultivations made from the mixture will only yield a growth resulting from the germination of the spores only.

Differential sterilisation at 80° C. is most conveniently carried out in a water-bath of special construction, designed by Balfour Stewart (Fig. 113). It consists of a double-walled copper vessel mounted on legs, and provided with a tubulure communicating with the space between the walls. This space is nearly filled with benzole (boiling-point 80° C.), and to the tubulure is fitted a long glass tube, some 2 metres long and about 0.75 cm. diameter, serving as a condensing tube. The interior of the vessel is partly filled with water and covered with a lid which is perforated for a thermometer. This latter dips into the water and records its temperature. A very small Bunsen flame under the apparatus suffices to keep the benzole boiling and the water within at a constant temperature of 80° C. The bath is thus always ready for use.

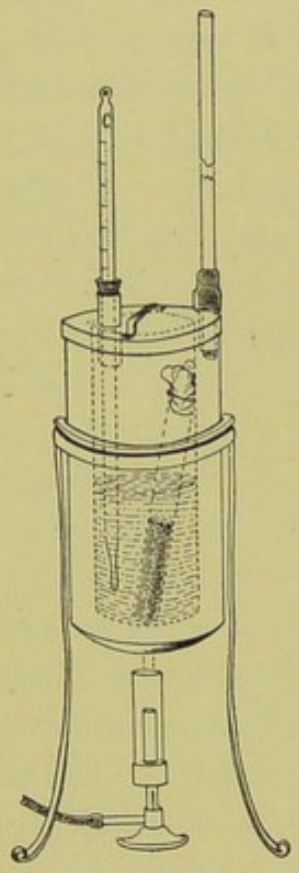


Fig. 113.—Benzole bath.

METHOD.—To use the apparatus,

1. Place some of the mixture itself, if fluid, containing the spores, or an emulsion of the same if derived from solid material, in a test-tube.

2. Immerse the test-tube in the water contained in the benzole bath, taking care that the upper level of the liquid in the tube is at least 2 cm. beneath the surface of the water in the copper vessel.

3. The temperature of the water, of course, falls a few degrees after opening the bath and introducing

a tube of colder liquid, but after a few minutes the temperature will have again reached 80° C.

4. When the thermometer again records 80° C., note the time, and fifteen minutes later remove the tube containing the mixture from the bath.

5. Make cultures upon suitable media; incubate.

7. Differential Atmosphere Cultivation.—

(*a*) By adapting the atmospheric conditions to the particular organism it is desired to isolate, it is comparatively easy to separate a strict aerobe from a strict anaerobe, and *vice versâ*. In the first case, however, it is important that the cultivations should be made upon solid media, for if carried out in fluid media the aerobes multiplying in the upper layers of fluid render the depths completely anaerobic, and under these conditions the growth of the anaerobes will continue unchecked.

(*b*) When it is desired to separate a facultative anaerobe from a strict anaerobe, it is generally sufficient to plant the mixture upon the sloped surface agar, incubate aerobically at 37° C., and examine carefully at frequent intervals. At the first sign of growth, subcultivations must be prepared and treated in a similar manner. As a result of these rapid subcultures, the facultative anaerobe will be secured in pure culture at about the third or fourth generation.

(*c*) If, on the other hand, the strict anaerobe is the organism required from a mixture of facultative and strict anaerobes, pour plates of glucose formate agar (or gelatine) in the usual manner, place them in a Bullock's or Novy's jar, and incubate at a suitable temperature. Pick off the colonies of the required organism when the growth appears, and transfer to tubes of the various media.

Incubate under suitable conditions as to temperature.

8. Animal Inoculation.—Finally, when dealing with

pathogenic organisms, it is often advisable to inoculate some of the impure culture (or even some of the original *materies morbi*) into an animal specially chosen on account of its susceptibility to the particular pathogenic organism it is desired to inoculate. Indeed, with some of the more sensitive and strictly parasitic bacteria this method of animal inoculation is practically the only method that will yield a satisfactory result.

XIV. METHODS OF IDENTIFICATION.

In order to identify an organism after nutrient media have been inoculated, and tube, plate, and other cultivations prepared, these are incubated under suitable conditions as to temperature and environment, are examined from time to time (a) **macroscopically,** (b) by **microscopical methods,** (c) by **chemical methods,** (d) by **physical methods,** (e) by **inoculation methods,** and the results of these examinations duly recorded.

It must be stated definitely that no micro-organism can be identified by any *one* character or property, whether microscopical, biological, or chemical, but that on the contrary its entire life history must be carefully studied and then its identity established from a consideration of the sum total of these observations.

In order to give to the recorded results their maximum value it is essential that they should be exact and systematical, therefore some such scheme as the following should be adhered to; and especially is this necessary in describing an organism not previously isolated and studied.

SCHEME OF STUDY.

Designation:

Originally isolated by .....in 18..., from .....

1. **Cultural Characters.**—(*Vide* Macroscopical Examination of Cultivation, page 207.)

Gelatine plates,
Gelatine streak,
Gelatine stab,
Gelatine shake,
} at 20° C.

Agar plates,
Agar streak or smear,
Agar stab,
Inspissated blood-serum,
Bouillon,
Litmus milk,
Potato,
} at 20° C. and 37° C.

Special media for the purpose of demonstrating characteristic reactions.

2. **Morphology.**—(*Vide* Microscopical Examination of Cultivations, page 218.)

Vegetative forms:

Shape.
Size.
Motility.
Flagella (if present).
Capsule (if present).
Involution forms.
Pleomorphism (if observed).

Sporing forms (if observed). Of which class?

Staining reactions.

3. **Biology.**—(*Vide* Physical Examination of Cultures, page 238.)

Vitality.

Resistance to lethal agents:

Physical:

Light.
Colours.

Chemical germicides.

Atmosphere.

Temperature.

Reaction of nutrient media.

Agglutination reaction.

4. **Chemical Products of Growth.**—(*Vide* Chemical Examination of Cultivations, page 221.)

Chromogenesis.

Photogenesis.

Enzyme formation.
Fermentation of carbohydrates:
In glucose gelatine shake cultivation.
In saccharose gelatine or bouillon.
In lactose gelatine or bouillon.
In maltose gelatine or bouillon.
In glycerine bouillon or bouillon.
Acid formation.
Alkali formation (if present).
Indol formation.
Phenol formation.
Reducing and oxidising agents.
Gas formation.

5. Pathogenicity:

Susceptible animals.
Immune animals.
Experimental inoculation, symptoms of disease.
Post-mortem appearances.
Virulence:
Length of time maintained.
Upon what medium?
Minimal lethal dose.
Is virulence readily exalted and attenuated?
Toxin formation.

MACROSCOPICAL EXAMINATION OF CULTIVATIONS.

In describing the naked-eye and low-power appearances of the bacterial growth the descriptive terms introduced by Chester (and included in the following scheme) should be employed.

Solid Media.

Plate Cultures.—

Gelatine.—Note the presence or absence of liquefaction of the surrounding medium. If liquefaction is present, note shape and character (*vide* page 215, "stab" cultures).

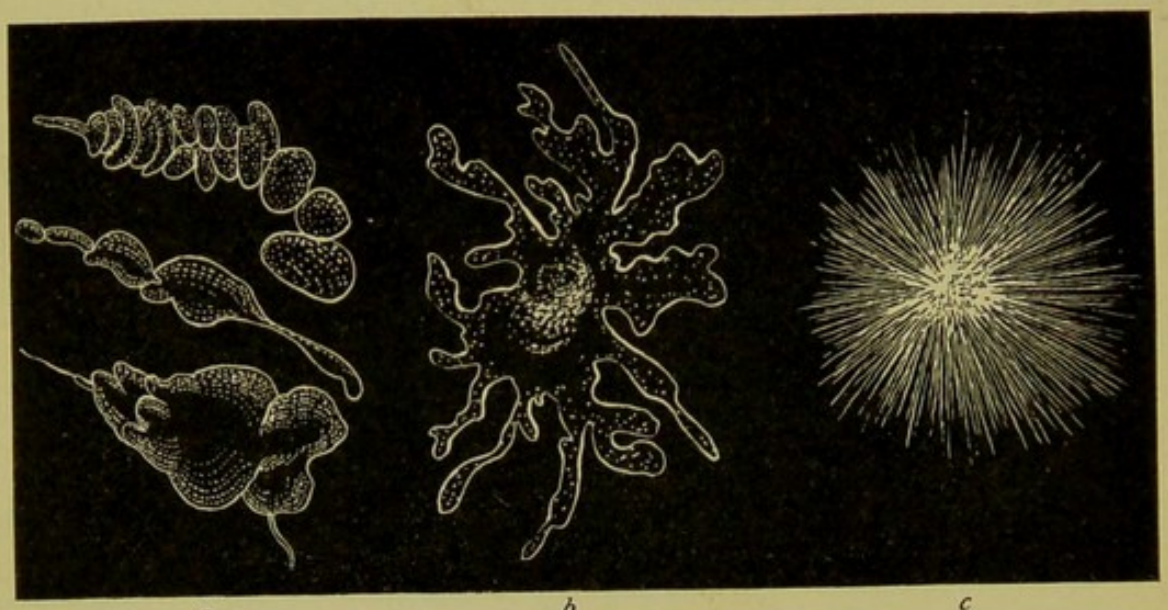
Agar.—No liquefaction takes place in this medium. The liquid found on the surface of the agar (or at the bottom of the tube in agar tube cultures) is merely water which has been expressed during solidification and has subsequently condensed.

Gelatine and Agar.—Examine the colonies at various intervals—

(a) With the naked eye.

(b) Under a low power (1 inch) of the microscope, or by means of a small dissecting microscope.

Distinguish superficial from deep colonies and note the characters of the individual colonies.



a *b* *c*

Fig. 114.—Types of colonies: *a*, Cochleate; *b*, amœboid; *c*, mycelioid.

(*A*) *Size.*—The diameter in millimetres, at the various ages.

(*B*) *Shape.*—

Punctiform: Dimensions too slight for defining form by naked eye; minute, raised, hemispherical.

Round: Of a more or less circular outline.

Elliptical: Of a more or less oval outline.

Irregular.

Fusiform: Spindle-shaped, tapering at each end.

Cochleate: Spiral or twisted like a snail shell (Fig. 114, *a*).

Amœboid: Very irregular, streaming (Fig. 114, *b*).

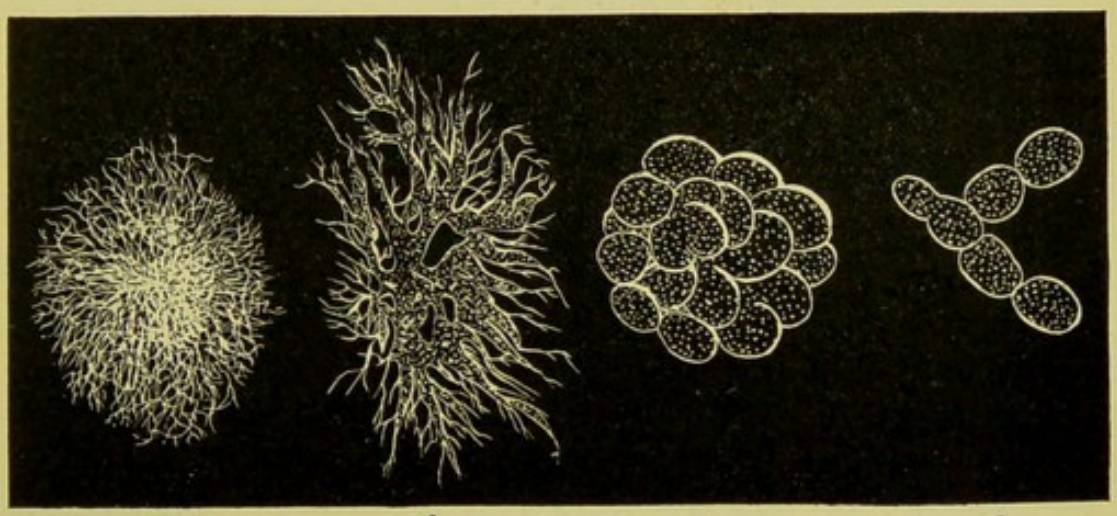
Mycelioid: A filamentous colony, with the radiate character of a mould (Fig. 114, *c*).

Filamentous: An irregular mass of loosely woven filaments (Fig. 115, *a*).

Floccose: Of a dense woolly structure.

Rhizoid: Of an irregular, branched, root-like character (Fig. 115, *b*).

Conglomerate: An aggregate of colonies of similar size and form (Fig. 115, *c*).



a *b* *c* *d*

Fig. 115.—Types of colonies: *a*, Filamentous; *b*, rhizoid; *c*, conglomerate; *d*, toruloid.

Toruloid: An aggregate of colonies, like the budding of the yeast plant (Fig. 115, *d*).

Rosulate: Shaped like a rosette.

(*C*) *Surface Elevation.*—

1. General Character of Surface as a Whole:

Flat: Thin, leafy, spreading over the surface (Fig. 116, *a*).

Effused: Spread over the surface as a thin, veily layer, more delicate than the preceding.

Raised: Growth thick, with abrupt terraced edges (Fig. 116, *b*).

Convex: Surface the segment of a circle, but very flatly convex (Fig. 116, *c*).

Pulvinate: Surface the segment of a circle, but decidedly convex (Fig. 116, *d*).

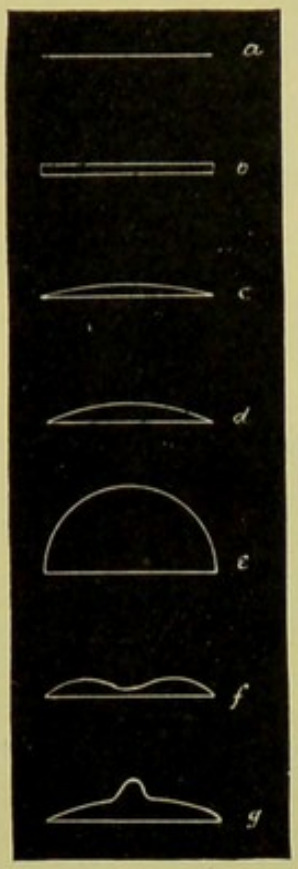


Fig. 116.—Surface elevation of colonies: *a*, Flat; *b*, raised; *c*, convex; *d*, pulvinate; *e*, capitate; *f*, umbilicate; *g*, umbonate.

Capitate: Surface hemispherical (Fig. 116, *e*).

Umbilicate: Having a central pit or depression (Fig. 116, *f*).

Umbonate: Having a central convex nipple-like elevation (Fig. 116, *g*).

2. Detailed Characters of Surface:

Smooth: Surface even, without any of the following distinctive characters.

Alveolate: Marked by depressions separated by thin walls so as to resemble a honeycomb (Fig. 117).

Punctate: Dotted with punctures like pin-pricks.

Bullate: Like a blistered surface, rising in convex prominences, rather coarse.

Vesicular: More or less covered with minute vesicles due to gas formation; more minute than bullate.

Verrucose: Wart-like, bearing wart-like prominences.

Squamose: Scaly, covered with scales.

Echinate: Beset with pointed prominences.

Papillate: Beset with nipple or mamma-like processes.

Rugose: Short irregular folds, due to shrinkage of surface growth.

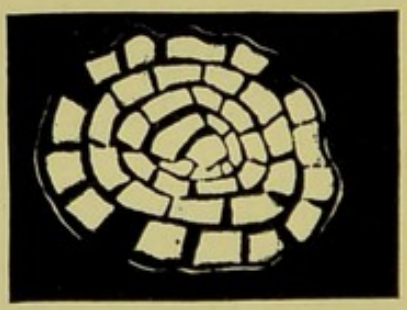


Fig. 117.—Types of colonies—alveolate.

Corrugated: In long folds, due to shrinkage.

Contoured: An irregular but smoothly undulating surface, like the surface of a relief map.

Rimose: Abounding in chinks, clefts, or cracks.

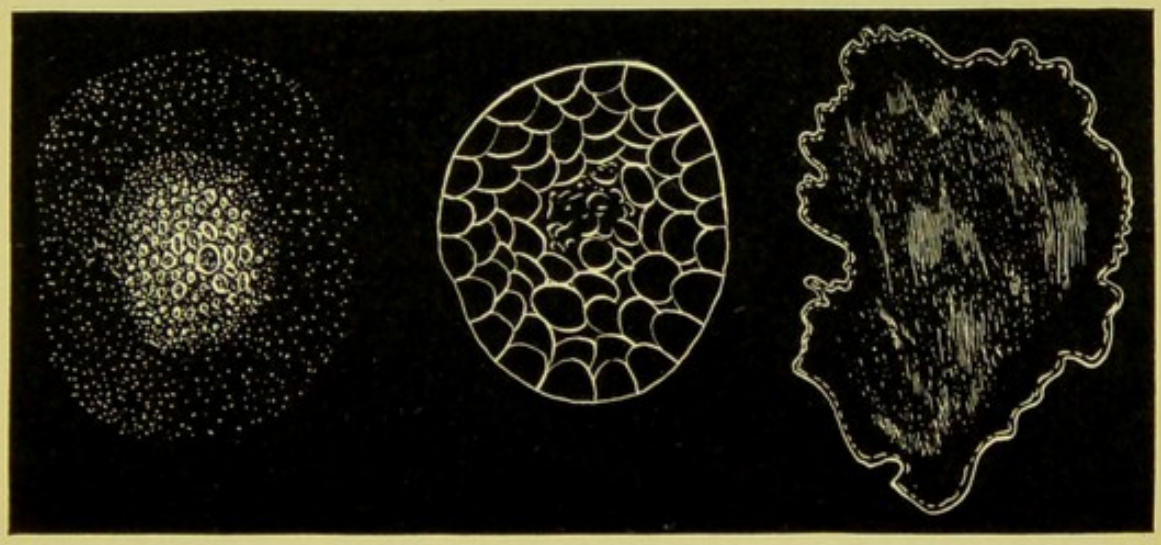
(D) Internal Structure of Colony (Microscopical).—

Refraction Weak: Outline and surface of relief not strongly defined.

Refraction Strong: Outline and surface of relief strongly defined; dense, not filamentous colonies.

1. General:

Amorphous: Without definite structure, as below specified.



a *b* *c*

Fig. 118.—Types of colonies: *a*, Grumose; *b*, moruloid; *c*, clouded.

Hyaline: Clear and colourless.

Homogeneous: Structure uniform throughout all parts of the colony.

Homochromous: Colour uniform throughout.

2. Granulations or Blotchings:

Finely granular.

Coarsely granular.

Grumose: Coarser than the preceding, with a clotted appearance, and particles in clustered grains (Fig. 118, *a*).

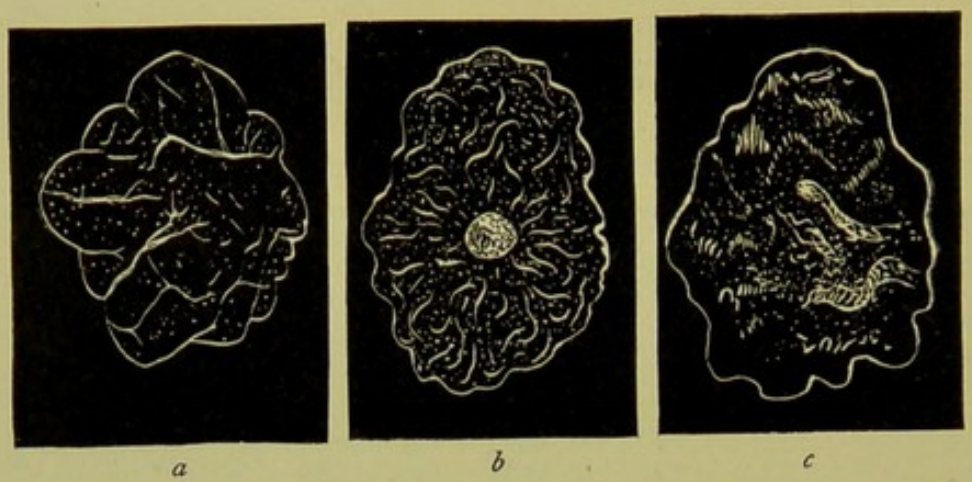
Moruloid: Having the character of a mulberry, seg-

mented, by which the colony is divided in more or less regular segments (Fig. 118, *b*).

Clouded: Having a pale ground, with ill-defined patches of a deeper tint (Fig. 118, *c*).

3. Colony Marking or Striping:

Reticulate: In the form of a network, like the veins of a leaf (Fig. 119, *a*).



a *b* *c*

Fig. 119.—Types of colonies: *a*, Reticulate; *b*, gyrose; *c*, marmorated.

Areolate: Divided into rather irregular, or angular, spaces by more or less definite boundaries.

Gyrose: Marked by wavy lines, indefinitely placed (Fig. 119, *b*).

Marmorated: Showing faint, irregular stripes, or traversed by vein-like markings, as in marble (Fig. 119, *c*).

Fig. 120.—Types of colonies—curled.

Rivulose: Marked by lines like the rivers of a map.

Rimose: Showing chinks, cracks, or clefts.

4. Filamentous Colonies:

Filamentous: As already defined.

Floccose: Composed of filaments, densely placed.

Curled: Filaments in parallel strands, like locks or ringlets, as in agar colonies of B. anthracis.

(*E*) *Edges of Colonies.*—

Entire: Without toothing or division (Fig. 121, *a*).

Undulate: Wavy (Fig. 121, *b*).

Repand: Like the border of an open umbrella (Fig. 121, *c*).

Erose: As if gnawed, irregularly toothed (Fig. 121, *d*).

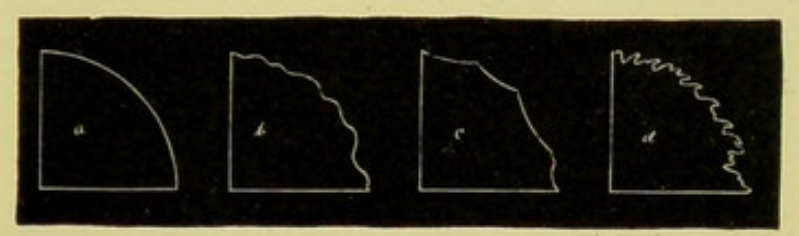


Fig. 121.—Edges of colonies: *a*, Entire; *b*, undulate; *c*, repand; *d*, erose.

Lobate.

Lobulate: Minutely lobate (Fig. 122, *e*).

Auriculate: With ear-like lobes (Fig. 122, *f*).

Lacerate: Irregularly cleft, as if torn (Fig. 122, *g*).

Fimbriate: Fringed (Fig. 122, *h*).

Ciliate: Hair-like extensions, radiately placed (Fig. 122, *j*).

Tufted.

Filamentous: As already defined.

Curled: As already defined.

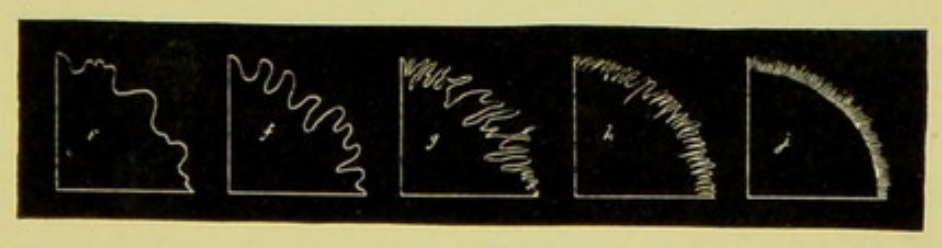


Fig. 122.—Edges of colonies: *e*, Lobar-lobulate; *f*, auriculate; *g*, lacerate; *h*, fimbriate; *j*, ciliate.

(*F*) *Optical Characters* (after Shuttleworth).—

1. General Characters:

Transparent: Transmitting light.

Vitreous: Transparent and colourless.

Oleaginous: Transparent and yellow; olive to linseed-oil coloured.

Resinous: Transparent and brown, varnish or resin-coloured.

Translucent: Faintly transparent.

Porcelaneous: Translucent and white.

Opalescent: Translucent; greyish-white by reflected light.

Nacreous: Translucent, greyish-white, with pearly lustre.

Sebaceous: Translucent, yellowish or greyish-white.

Butyrous: Translucent and yellow.

Ceraceous: Translucent and wax-coloured.

Opaque.

Cretaceous: Opaque and white, chalky.

Dull: Without lustre.

Glistening: Shining.

Fluorescent.

Iridescent.

2. Chromogenicity:

Colour of pigment.

Pigment restricted to colonies.

Pigment restricted to medium surrounding colonies.

Pigment present in colonies and in medium.

Streak or Smear Cultures.—

Gelatine and Agar.—Note general points as indicated under plate cultivations.

Inspissated Blood-serum.—Note the presence or absence of liquefaction of the medium. (The presence of condensation water at the bottom of the tube must not be confounded with liquefaction of the medium.)

All Oblique Tube Cultures.—

1. Colonies Discrete: Size, shape, etc., as for plate cultivations (*vide* page 208).

2. Colonies Confluent: Surface elevation and character of edge, as for plate cultivations (*vide* page 209).

Chromogenicity: As for plate cultures (*supra*).

Gelatine Stab Cultures.—

(*A*) *Surface Growth.*—As for individual colonies in plate cultures (*vide* page 209).

(*B*) *Line of Puncture.*—

Filiform: Uniform growth, without special characters (Fig. 123, *a*).

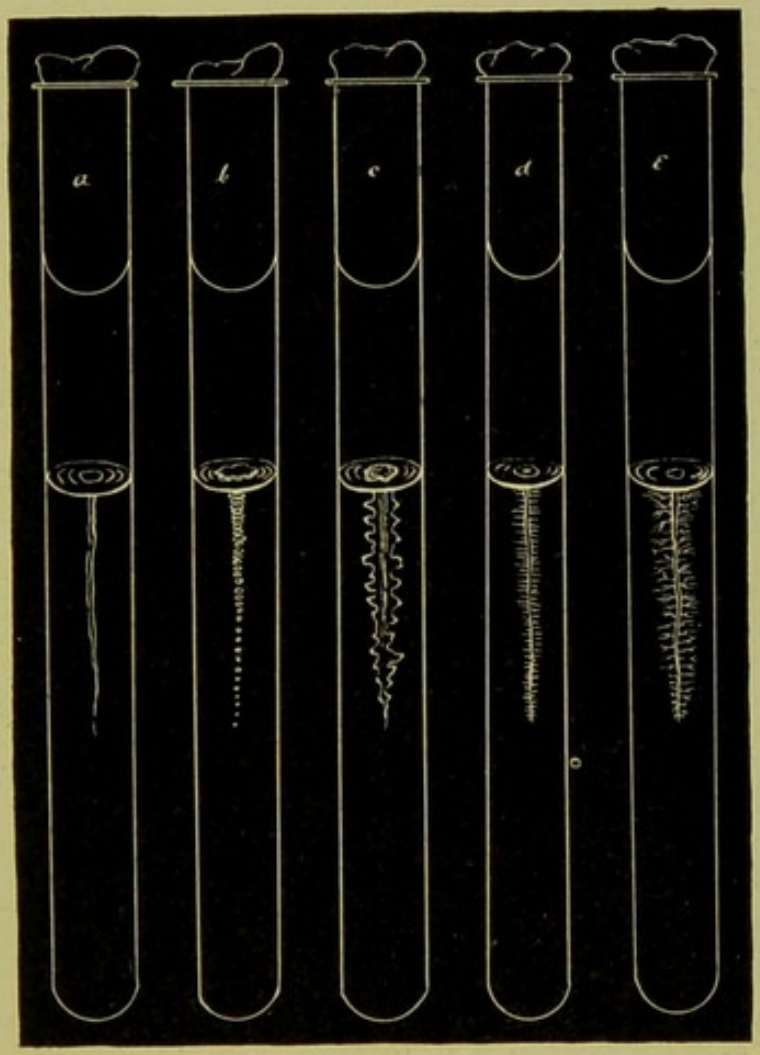


Fig. 123.—Stab cultivations—types of growth: *a*, Filiform; *b*, beaded; *c*, echinate; *d*, villous; *e*, arborescent.

Nodose: Consisting of closely aggregated colonies.

Beaded: Consisting of loosely placed or disjointed colonies (Fig. 123, *b*).

Papillate: Beset with papillate extensions.

Echinate: Beset with acicular extensions (Fig. 123, *c*).

Villous: Beset with short, undivided, hair-like extensions (Fig. 123, *d*).

Plumose: A delicate feathery growth.

Arborescent: Branched or tree-like, beset with branched hair-like extensions (Fig. 123, *e*).

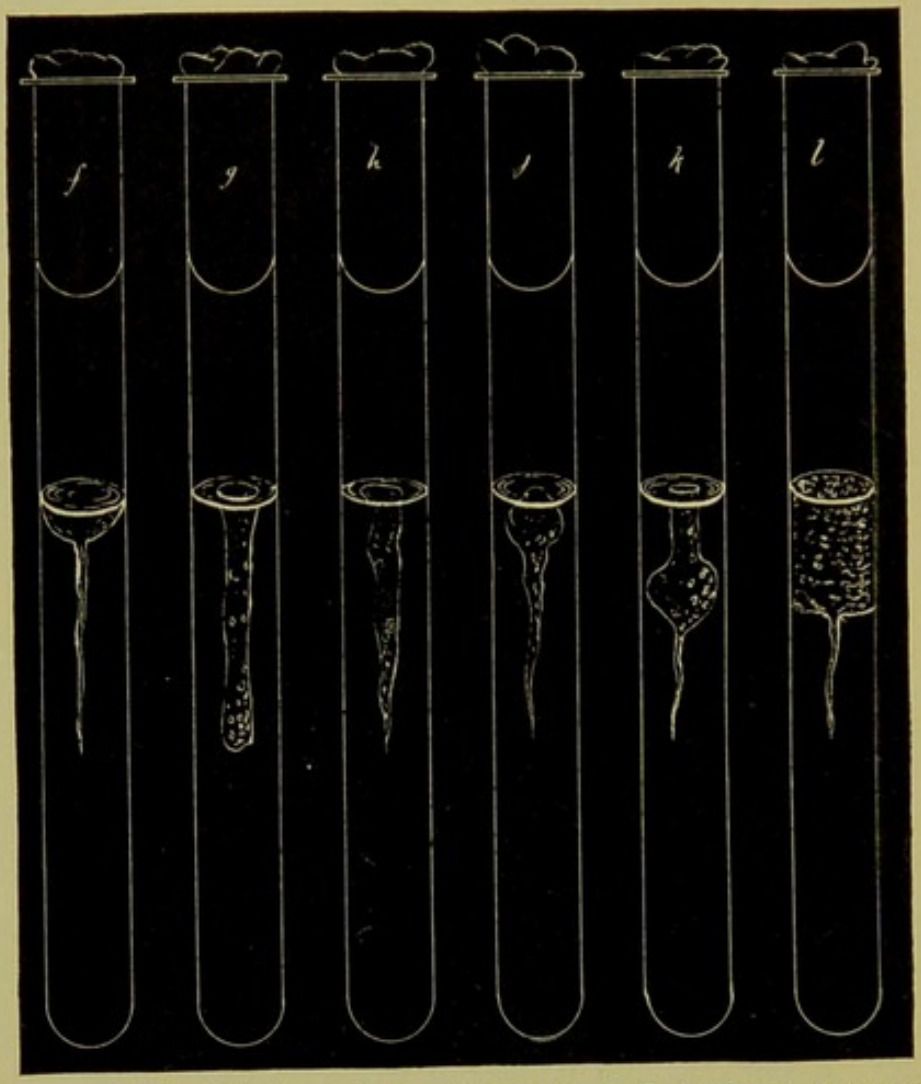


Fig. 124.—Stab cultivations—types of growth: *f*, Crateriform; *g*, saccate; *h*, infundibuliform; *j*, napiform; *k*, fusiform; *l*, stratiform.

(*C*) *Area of Liquefaction* (if present).—

Crateriform: A saucer-shaped liquefaction of the gelatine (Fig. 124, *f*).

Saccate: Shape of an elongated sack, tubular, cylindrical (Fig. 124, *g*).

Infundibuliform: Shape of a funnel, conical (Fig. 124, *h*).

Napiform: Shape of a turnip (Fig. 124, *j*).

Fusiform: Outline of a parsnip, narrow at either end, broadest below the surface (Fig. 124, *k*).

Stratiform: Liquefaction extending to the walls of the tube and downwards horizontally (Fig. 124, *l*).

(*D*) *Character of the Liquefied Gelatine.*—

1. Pellicle on surface.
2. Uniformly turbid.
3. Granular.
4. Mainly clear, but containing flocculi.
5. Deposit at apex of liquefied portion.

(*E*) *Production of Gas Bubbles.*

Shake Cultures.—

1. Presence or absence of liquefaction.
2. Production of gas bubbles.
3. Bulk of growth at the surface—aerobic.
4. Bulk of growth in depths—anaerobic.

Fluid Media.

1. Surface of the Liquid.—

Presence or absence of froth due to gas bubbles.
Presence or absence of pellicle formation.
Character of pellicle.

2. Body of the Liquid.—

Uniformly turbid.
Flocculi in suspension.
Granules in suspension.
Clear, with precipitate at bottom of tube.
Colouration of fluid, presence or absence of.

3. Precipitate.—

Character.
Amount.
Colour.

Litmus Milk Cultivations.—

1. Reaction: { Unaltered. / Acid. / Alkaline.

2. Odour.
3. Formation of gas.
4. Consistency: { Unaltered. / Digested (? character of solution). / Coagulated. }
5. Clot: Character { hard: solid. / soft: flocculent. }
   (*a*) Coagulum undissolved.
   (*b*) Coagulum finally digested, completely: incompletely.
   Resulting solution, clear: turbid.
6. Whey: { Abundant. / Scanty. / Clear. / Turbid. }

BY MICROSCOPICAL METHODS.

Preparations must be made from the cultivations at intervals of, say, twenty-four hours, during the period they are under observation, and examined—

(A) **Living.—1.** In **hanging drop,** to determine *motility* or *non-motility.*

In this connection it must be remembered that under certain conditions as to environment (*e. g.*, cold, heat, light, unsuitable medium, etc.) motile bacilli may fail to exhibit activity. No organism, therefore, should be recorded as non-motile from one observation only; a series of observations at different ages and under varying conditions should form the basis of an opinion as to the absence of true locomotion.

Size.—In the case of non-motile or sluggishly motile organisms, endeavour to measure several individuals in each hanging drop by means of the eyepiece micrometer, and average the results.

If the organism is one which forms spores, observe—

(*a*) *Spore Formation.*—Prepare hanging-drop cultivations (*vide* page 69) from vegetative forms of the organism, adding a trace of magenta solution (0.5 per cent.) to the drop, on the point of the platinum needle, to facilitate the observation of the phenomenon by rendering the bacilli more distinct.

Place the preparation on the stage of the microscope; if necessary, using a warm stage.

Arrange illumination, etc., and select a solitary bacillus for observation, by the help of the $\frac{1}{6}$-inch lens.

Substitute the $\frac{1}{12}$-inch oil-immersion lens for the sixth, and observe the formation of the spore; if possible, measure any alteration in size which may occur by means of the Ramsden micrometer.

(*b*) *Spore Germination.*—In a similar manner prepare hanging-drop cultivations from old cultivations in which no living vegetative forms are present, and observe the process of germination.

The comfort of the microscopist is largely enhanced in those cases where the period of observation is at all lengthy, by the use of some form of eye screen before the unemployed eye, such as is figured on page 59 (Fig. 41).

If it is impossible to carry out the method suggested above, proceed as follows:

(*a*) *Spore Formation.*—Plant the organism in broth and incubate under optimum conditions.

At regular intervals, say every thirty minutes, remove a loopful of the cultivation and prepare a cover-slip film preparation.

Fix, while still wet, in the corrosive sublimate fixing solution.

Stain with aniline gentian violet, and partially decolourise with 2 per cent. acetic acid.

Mount and number consecutively.

(*b*) *Spore Germination.*—Expose a thick emulsion of

the spores to a temperature of 80° C. for ten minutes in the differential steriliser (*vide* page 202).

Transfer the emulsion to a tube of sterile nutrient broth and incubate.

Remove specimens from the tube culture at intervals of, say, five minutes.

Fix, stain, etc., as under (*a*), and examine.

(B) **Fixed.—2. In stained preparations.**

(*a*) To determine points in *morphology:*

Shape (*vide* classification, page 111).

Size:

(a) Prepare cover-slip film preparations at the various ages, and fix by exposure to a temperature of 115° C. for twenty minutes (*vide* page 75).

(b) Stain the preparations by Gram's method (if applicable) or with dilute carbol-fuchsin, and mount in the usual way.

(c) Measure (*vide* page 63) some twenty-five individuals in each film by means of the Ramsden's or the stage micrometer and average the result.

Pleomorphism: If noted, record—

The predominant character of the variant forms.

On what medium or media they are observed.

(*b*) To demonstrate details of *structure:*

Flagella: If noted, record—

Method of staining (*vide* page 87).

Position and arrangement (*vide* page 115).

Number.

Spores: If noted, record—

Method of staining.

Shape.

Size.

Position within the parent cell.

Condition, as to shape, of the parent cell (*vide* page 74).

On what medium they are best observed.

Age of medium.

Conditions of environment as to temperature, atmosphere.

Method of germination (*vide* page 119).

Involution Forms: If noted, record—

Method of staining.

Character (*e. g.*, if living or dead).

Shape.

On what medium they are observed.

Age of medium.

Environment.

Metachromatic Granules: If noted, record—

Method of staining.

Character of granules.

Number of granules.

Colour of granules.

3. Staining Reactions.—

1. *Gram's Method.*—Decolourised or retain the stain.

2. *Neisser's Method.*—If granules are noted, record—

1. Position.
2. Number.

3. *Ziehl-Neelsen's Method.*—Decolourised or acid-fast.

4. *Simple Aniline Dyes.*—(Noting those giving the best results, with times of staining.)

Methylene-blue
Fuchsin
Gentian violet
Thionine blue
} and their modifications.

BY CHEMICAL METHODS.

Test cultivations of the organism for the presence of—

Soluble enzymes—proteolytic, diastatic, invertin.

Organic acids—(*a*) quantitatively—*i. e.*, estimate the total acid production; (*b*) qualitatively for formic, acetic, propionic, butyric, lactic.

Ammonia.

Alcohol—ethyl alcohol, aldehyde, acetone.

Aromatic products—indol, phenol.

Soluble pigments.

Test the power of reducing (*a*) colouring matters, (*b*) nitrates to nitrites.

Investigate the gas production—H_2S, CO_2, H. Estimate the ratio between the last two gases.

Prepare all cultivations for these methods of examination under *optimum* conditions, previously determined for each of the organisms it is intended to investigate, as to

(*a*) Reaction of medium;
(*b*) Incubation temperature;
(*c*) Atmospheric environment.

and keep careful records of these points, and also of the age of the cultivation used in the final examination.

Examine the cultivations for the various products of bacterial metabolism after forty-eight hours' growth, and never omit to examine "control" (uninoculated) tube or flask of medium kept for a similar period under identical conditions. If the results are negative, test further cultivations at three days, five days, and ten days.

1. Enzyme Production.—

(*A*) *Proteolytic Enzymes.*—(Convert proteids into peptones and propeptones; *e. g.*, B. pyocyaneus.)

Media Required:

Blood-serum and milk-serum which have been carefully filtered through a porcelain candle.

Reagents Required:

Ammonium sulphate.

$\frac{n}{10}$ caustic soda solution.

Copper sulphate, 1 per cent. aqueous solution.

METHOD.—

1. Prepare cultivations in bulk (50 c.c.) in a flask and incubate.

2. Add 60 grammes of ammonium sulphate to 40 c.c. of the cultivation, and warm to 50° C. for half an hour. (This precipitates the proteid bodies.)

3. Filter.

Test the filtrate for propeptones and peptones.

Make the filtrate strongly alkaline with caustic soda.

Add a few drops of copper sulphate solution.

Violet colour = peptones.

(*B*) *Diastatic Enzymes.*—(Convert starch into sugar; *e. g.*, B. subtilis.)

Medium Required:
Inosite-free bouillon.
Reagents Required:
Starch.
Thymol.
Fehling's solution.

METHOD.—

1. Prepare tube cultivation and incubate.

2. Prepare a thin starch paste and add 2 per cent. thymol to it.

3. Mix equal parts of the cultivation to be tested and the starch paste, and place in the incubator at 37° C. for six to eight hours.

4. Filter.

Test the filtrate for sugar.

Boil some of the Fehling's solution in a test-tube.

Add the filtrate drop by drop until, if necessary, a quantity has been added equal in amount to the Fehling's solution employed, keeping the mixture at the boiling-point during the process.

Yellow or orange precipitate = sugar.

(*C*) *Invertin Enzymes.*—(Convert saccharose into glucose; *e. g.*, B. fluorescens liquefaciens.)

Medium Required:
Inosite-free bouillon.
Reagents Required:
Cane sugar, 2 per cent. aqueous solution.
Carbolic acid.

METHOD.—

1. Prepare tube cultivations and incubate.

2. Add 2 per cent. of carbolic acid to the sugar solution.

3. Mix equal quantities of the carbolised sugar solution and the cultivation in a test-tube; allow the mixture to stand for several hours.

4. Filter.

Test the filtrate for glucose as in the preceding section.

(*D*) *Rennet and "Lab" Enzymes.*—(Coagulate milk independently of the action of acids; *e. g.*, B. prodigiosus.)

Media Required:
Inosite-free bouillon.
Litmus milk.

METHOD.—

1. Prepare tube cultivations and incubate.

2. After incubation heat the cultivation to 55° C. for half an hour, to sterilise.

3. By means of a sterile pipette run 5 c.c. of the cultivation into each of three tubes of litmus milk.

4. Place in the cold incubator at 22° C. and examine each day for ten days.

Absence of coagulation at the end of that period will indicate absence of rennet ferment formation.

2. Acid Production.—

(*a*) *Quantitative.*—

Medium Required:
Sugar (glucose) bouillon (*vide* page 142) of known "optimum" reaction.
Apparatus and Reagents Required:
As for estimating reaction of media (*vide* page 129).

METHOD.—

1. Prepare cultivation in bulk (100 c.c.) in a flask.

2. After suitable incubation, heat in the steamer at 100° C. for thirty minutes to sterilise.

3. Determine the *titre* of the medium as described in the preparation of nutrient media (*vide* page 130).

4. The difference between the original titre of the medium and that now estimated gives the total acid production in terms of normal NaOH.

NOTE.—If the growth is very heavy it may be a difficult matter to determine the end-point. The cultivation should then be filtered through a Berkfeld filter candle previous to step 2, and the filtrate employed in the titration.

(*b*) *Qualitative* (of all the organic acids present).—

Medium Required:

Sugar (glucose or lactose) bouillon as in quantitative examination.

Reagents Required:

Hydrochloric acid, concentrated.
Sulphuric acid, concentrated (pure).
Sulphuric acid, 25 per cent. solution.
Ammonia.
Ammonium sulphide.
Baryta water.
Sodium carbonate, saturated aqueous solution.
Absolute alcohol.
Ether.
Calcium chloride.
Zinc oxide.
Permanganate of potash, 4 per cent. aqueous solution.
Schiff's reagent.
Arsenious oxide.
Ferric chloride, 4 per cent. aqueous solution.
Cobalt nitrate, 2 per cent. aqueous solution.
Silver nitrate, 1 per cent. aqueous solution.
Lugol's iodine (*vide* page 94).
Cane sugar, 10 per cent. aqueous solution.
Hard paraffin wax (melting-point about 52° C.).

METHOD.—

1. Prepare cultivation in bulk (500 c.c.) in a litre flask and add sterilised precipitated chalk, 10 grammes. Incubate at the optimum temperature.

2. After incubation throw a piece of paraffin wax (about a centimetre cube) into the cultivation and connect up the flask with a condenser.

The paraffin, which liquefies and forms a thin layer on the surface of the fluid, is necessary to prevent the cultivation frothing up and running unaltered through the condenser during the subsequent process of distillation.

3. Distil over 200 to 300 c.c.

Use a rose-top burner to minimise the danger of

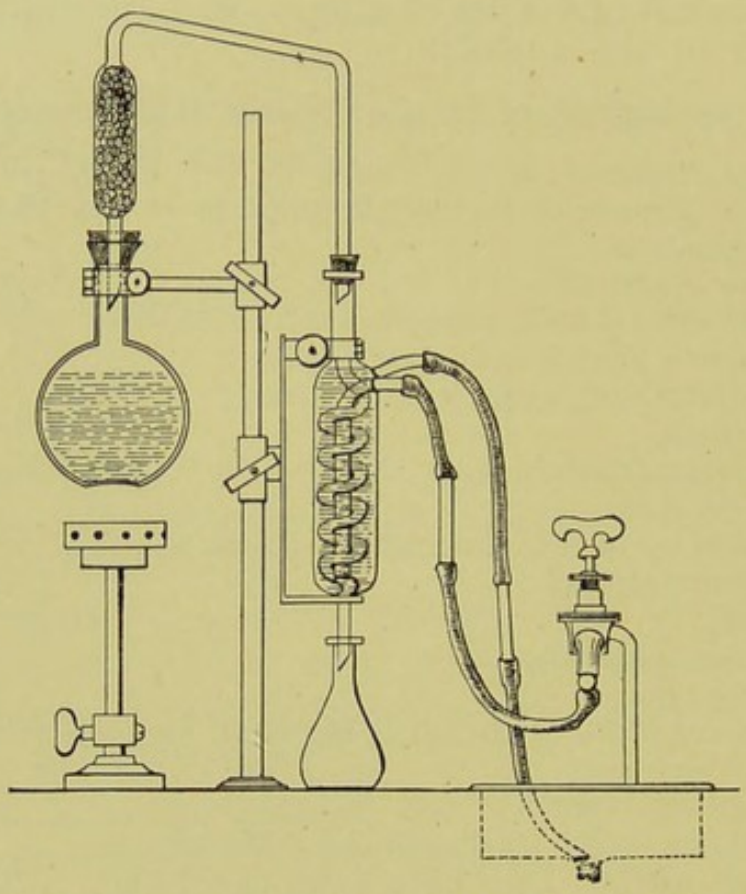


Fig. 125.—Arrangement of distillation apparatus for acids, etc.

cracking the flask; and to the same end, well agitate the contents of the flask to prevent the chalk settling.

The distillate "A" will contain alcohol, etc. (*vide* page 229); the residue "a" will contain the volatile and fixed acids.

4. Disconnect the flask and filter the chalk from the contained residue; add 10 c.c. conc. hydrochloric acid to the filtrate; mix well.

5. Precipitate the calcium by adding sodium carbonate solution, until alkaline.

6. Boil thoroughly (to ensure *complete* precipitation of lime) and filter.

7. Add 20 c.c. sulphuric acid (25 per cent.) to the filtrate (this liberates the volatile acids), and distil as completely as possible.

DISTILLATE "B."

(Volatile Acids.)

1. Saturate with baryta water to alkalinity, and evaporate to dryness.
2. Add 20 c.c. absolute alcohol and allow to stand, with frequent stirring, for two to three hours.
3. Filter and wash with alcohol.

FILTRATE
may contain barium propionate, barium butyrate.

1. Evaporate to dryness.
2. Dissolve residue in 150 c.c. water.
3. Saturate with calcium chloride.
4. Distil.
5. Test distillate for butyric acid:
   Add 3 c. c. alcohol and 4 drops concentrated sulphuric acid.
   Smell of pineapple = *butyric* acid.

Propionic acid in small quantities cannot be distinguished from butyric acid by tests within the scope of the bacteriological laboratory.

RESIDUE
may contain barium acetate, barium formate.

1. Evaporate off alcohol and dissolve up the residue on the filter in hot water.
2. Divide the solution into four portions:
   (a) Add ferric chloride solution.
   Brown colour = *acetic* or *formic* acids.
   (b) Add silver nitrate solution.
   White flocculent precipitate (soluble in hot water and separating in spangles when cool) = *acetic* acid.
   Add silver nitrate solution; then add one drop ammonia water, and boil.
   Black precipitate of metallic silver = *formic* acid.
   (c) Evaporate to dryness; mix with equal quantity of arsenious oxide and heat on platinum foil.
   Unpleasant **smell of cacodyl** = *acetic* acid.
   (d) Add a few drops of mercuric chloride solution in test-tube, and heat to 70° C.
   Precipitate of mercurous chloride and the formation, after a long time, of a **metallic mirror** = *formic* acid.

The distillate "B" may contain acetic, propionic, formic, or butyric acid.

The residue "b" may contain lactic, oxalic, succinic, glycocholic, taurocholic, cholic, benzoic, hippuric, tannic, or gallic acid.

RESIDUE "b."

(Fixed Acids.)

1. Evaporate the remainder of the residue to a syrup.
2. Extract with ether by agitation in a separatory funnel. (This dissolves out the fixed organic acids.)
3. Evaporate ethereal extract to a syrup. (No residue = absence of lactic, oxalic, succinic acids.)
4. Add 100 c.c. water and mix thoroughly.
5. Add excess of zinc oxide and heat nearly to boiling. Filter.

FILTRATE

(a) Test for cholic acid series :
1. To 6 c.c. filtrate add 4 c.c. conc. sulphuric acid ; then add 1 drop cane sugar solution and warm to 75° C.
Crimson colour = *glycocholic*, *taurocholic*, or *cholic* acids.
2. To a portion of the filtrate add Lugol's iodine.
Blue colour (resembling that of the starch-iodo compound) = *cholic* acid.

(b) Test for lactic acid :
1. Acidify with hydrochloric acid.
2. Add ammonia water in slight excess and boil off the excess.
3. Add cobalt nitrate solution.
Violet colour (or if in sufficient quantity crystalline precipitate) = *lactic* acid.

Or—
1. Evaporate bulk of filtrate to dryness.
2. Dissolve in 10 c.c. hot water.
3. Allow to crystallise—with concentration if necessary.
Crystals of zinc lactate = *lactic* acid.
(Do not confuse with zinc sulphate, which will probably be present also.)

RESIDUE.

Dissolve in hydrochloric acid from off the filter.

(a) Test for oxalic acid :
1. Neutralise with ammonia till faintly alkaline.
2. Add calcium chloride solution.
White precipitate = *calcium oxalate*. No precipitate = absence of oxalic acid.

(b) Test for succinic, benzoic, or hippuric, salicylic, tannic, or gallic acid :
1. Neutralise with ammonia and boil off the excess.
2. Add ferric chloride solution on a glass rod.
Red-brown colouration or precipitate = *succinic* acid.
Buff colouration or precipitate = *benzoic* or *hippuric* acid.
Violet colouration or precipitate = *salicylic* acid.
Inky-black colouration or precipitate = *tannic* or *gallic* acid.

8. Use 50 c.c. of the distillate "B" for titrations. This will give the amount of volatile acid formation.

3. Ammonia Production.—

Medium Required:
Nutrient bouillon.
Reagent Required:
Nessler reagent.

METHOD.—

1. Prepare cultivation in bulk (100 c.c.) in a 250 c.c. flask and incubate together with a control flask.

Test the cultivation and the control for ammonia in the following manner:

2. To each flask add 2 grammes of calcined magnesia, then connect up with condensers and distil.

3. Collect 50 c.c. distillate, from each, in a Nessler glass.

4. Add to each 1 c.c. Nessler reagent by means of a clean pipette.

A yellow colour = ammonia.

The depth of colour is proportionate to the amount present.

4. Alcohol, etc., Production.—Divide the distillate "A" obtained in the course of a previous experiment (*vide* page 226, step 3) into four portions and test for the production of alcohol, acetaldehyde, acetone.

1. Add Lugol's iodine, then a little NaOH solution, and stir with a glass rod.

Pale yellow crystalline precipitate of iodoform, with its characteristic smell, indicates alcohol, aldehyde, or acetone.

The precipitate may be absent even when the odour is pronounced.

2. Add Schiff's reagent.

Violet or red colour = aldehyde.

3. To 10 c.c. of solution add 5 c.c. conc. sulphuric acid, and 1 c.c. of potassium permanganate solution. After an interval of five minutes add Schiff's reagent.

Red colour (due to oxidation of magenta in reagent) = aldehyde from alcohol.

4. Make the solution strongly alkaline with ammonia. Add gradually a solution of iodine in ammonium iodide. A black precipitate of nitrogen iodide forms, which quickly disappears on shaking. As soon as the precipitate tends to become permanent, it will change to iodoform if acetone is present.

5. Indol Production.—

Media Required:
Inosite-free bouillon (*vide* page 141).
Or peptone water (*vide* page 168).
Reagents Required:
Sulphuric acid, concentrated pure.
Sodium nitrite, 0.01 per cent. aqueous solution.

METHOD.—

1. Prepare several test-tube cultivations of the organism to be tested, and incubate.

Test for indol by means of the nitroso-indol reaction, in the following manner. (If the culture has been incubated at 37° C., it must be allowed to cool to the room temperature before applying the test.)

2. Remove the cotton-wool plug from the tube, and run in 1 c.c. sulphuric acid by means of a sterile pipette. Place the tube upright in a rack, and allow it to stand, if necessary, for ten minutes.

A rose-pink or red colour = indol (*plus a nitrite*).

3. If the colour of the medium remains unaltered, add 2 c.c. sodium nitrite solution, and again allow the culture to stand for ten minutes.

Red colouration = indol.

NOTE.—In place of performing the test in two stages as given above, 2 c.c. concentrated *commercial* sulphuric, hydrochloric, or nitric acid, all of which hold a trace of nitrite in solution, may be run into the cultivation. The development of a red colour within twenty minutes will indicate the presence of indol.

5a. Phenol Production.—

Medium Required:
Nutrient bouillon.
Reagents Required:
Hydrochloric acid, concentrated.
Millon's reagent.
Ferric chloride, 1 per cent. aqueous solution.

METHOD.—

1. Prepare cultivation in a Bohemian flask containing at least 50 c.c. of medium, and incubate.

Test for phenol in the following manner:

2. Add 5 c.c. hydrochloric acid to the cultivation and connect up the flask with a condenser.

3. Distil over 15 to 20 c.c. Divide the distillate into two portions.

4. Test one portion by adding 0.5 c.c. Millon's reagent and boiling.

A red colour = phenol.

5. Test the other portion by adding about 0.5 c.c. ferric chloride solution.

A violet colour = phenol.

NOTE.—If both indol and phenol appear to be present in cultivations of the same organism, it is well to separate them before testing. This may be done in the following manner:

1. Prepare inosite-free bouillon cultivation, say 200 or 300 c.c., in a flask as before.

2. Add 50 to 60 c.c. hydrochloric acid and connect up the flask with a condenser.

3. Distil over 50 to 70 c.c.

Distillate will contain both indol and phenol.

4. Render the distillate strongly alkaline with caustic potash and redistil.

Distillate will contain indol; residue will contain phenol.

5. Test the distillate for indol (*vide ante*).

6. Saturate the residue, when cold, with carbon dioxide and redistil.

7. Test this distillate for phenol (*vide ante*).

6. Pigment Production.—

1. Prepare tube cultivations upon the various media and incubate under varying conditions as to temperature (at 37° C. and at 20° C.), atmosphere (aerobic and anaerobic), and light (exposure to and protection from).

Note the conditions most favorable to pigment formation.

2. Note the solubility of the pigment in various solvents, such as water, hot and cold, alcohol, ether, chloroform, benzol, carbon bisulphide.

3. Note the effect of acids and alkalies respectively upon the pigmented cultivation, or upon solutions of the pigment.

4. Note spectroscopic reactions.

7. Reducing Agent Formation.—

(*a*) *Colour Destruction.—*

1. Prepare tube cultivations in nutrient bouillon tinted with litmus, rosolic acid, neutral red, and incubate.

2. Examine the cultures each day and note whether any colour change occurs.

(*b*) *Nitrates to Nitrites.—*

Medium Required:
Nitrate bouillon (*vide* page 143).
Or nitrate water (*vide* page 169).
Reagents Required:
Sulphuric acid (25 per cent.).
Metaphenylene diamine, 5 per cent. aqueous solution.

METHOD.—

1. Prepare tube cultivations and incubate together with control tubes (*i. e.*, uninoculated tubes of the same medium, placed under identical conditions as to environment).

This precaution is necessary as the medium is liable to take up nitrites from the atmosphere, and an opinion as to the absence of nitrites in the cultivation is often based upon an equal colouration of the medium in the control tube.

Test both the culture tube and the control tube for the presence of nitrites.

2. Add a few drops of sulphuric acid to the medium in each of the tubes.

3. Then run in 2 or 3 c.c. metaphenylene diamine into each tube.

A brownish-red colour = nitrites.

The depth of colour is proportionate to the amount present.

8. Gas Production.—

(A) Carbon Dioxide and Hydrogen.—

Apparatus Required:

Fermentation tubes (*vide* page 24) containing sugar bouillon (glucose, lactose, etc.). The medium should be prepared from inosite-free bouillon (*vide* page 141).

Reagent Required:

$\frac{n}{2}$ caustic soda.

METHOD.—

1. Inoculate the surface of the medium in the bulb of a fermentation tube and incubate.

2. Mark the level of the fluid in the closed branch of the fermentation tube, at intervals of twenty-four hours, and when the evolution of gas has ceased, measure the length of the column of gas with the millimetre scale.

Express this column of gas as a percentage of the entire length of the closed branch.

3. To analyse the gas and to determine roughly the relative proportions of CO_2 and H, proceed as follows:

Fill the bulb of the fermentation tube with caustic soda solution.

Close the mouth of the bulb with a rubber stopper.

Alternately invert and revert the tube six or eight times, to bring the soda solution into intimate contact with the gas.

Return the residual gas to the end of the closed branch, and measure.

The loss in volume of gas = carbon dioxide.

The residual gas = hydrogen.

Transfer gas to the bulb of the tube, and explode it by applying a lighted taper.

(*B*) *Sulphuretted Hydrogen.*—

Media Required:

Peptone iron solution (*vide* page 168).

Peptone lead solution.

1. Inoculate tubes of media, and incubate together with control tubes.

2. Examine from day to day, at intervals of twenty-four hours.

The liberation of the H_2S will cause the yellowish-white precipitate to darken to a brownish-black, or jet black, the depth of the colour being proportionate to the amount of sulphuretted hydrogen present.

Quantitative: For exact quantitative analyses of the gases produced by bacteria from certain media of definite composition, the methods devised by Pakes must be employed, as follows:

Apparatus Required:

Bohemian flask (300 to 1500 c.c. capacity) containing from 100 to 400 c.c. of the medium. The mouth of the flask is fitted with a perforated rubber stopper, carrying an L-shaped piece of glass tubing (the short arm passing just through the stopper). To the long arm of the tube is attached a piece of pressure tubing some 8 cm. in length, plugged at its free end with a piece of cotton-wool. Measure accurately the total capacity of the flask and exit tube, also the amount of medium contained. Note the difference.

Gas receiver. This is a bell jar of stout glass, 14 cm. high and 9 cm. in diameter. At its apex a glass tube is fused

in. This rises vertically 5 cm., and is then bent at right angles, the horizontal arm being 10 cm. in length. A three-way tap is let horizontally into the vertical tube just above its junction with the bell jar.

An iron cylinder just large enough to contain the bell jar.

About 15 kilos of mercury.

Melted paraffin.

An Orsat-Lunge working with mercury instead of water, provided with two gas tubes of extra length (capacity 120 and 60 c.c. respectively and graduated throughout, both being water-jacketed) or other gas analysis apparatus, capable of dealing with CO, CO_2, O, H, and N.

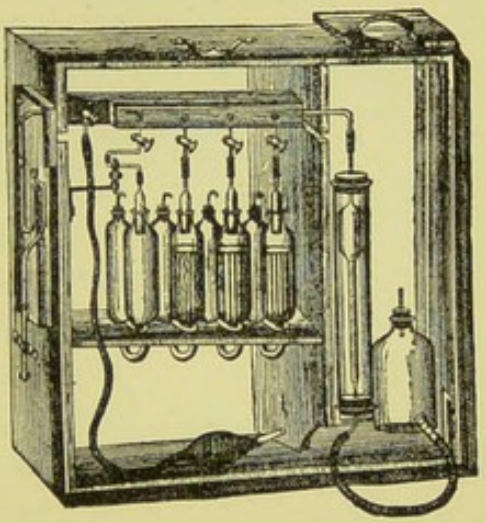


Fig. 126.—Orsat-Lunge gas analysis apparatus.

METHOD.—

1. Inoculate the medium in the flask in the usual manner, by means of a platinum needle, taking care that the neck of the flask and the rubber stopper are thoroughly heated in the flame before and after the operation.

2. Fill the iron cylinder with mercury.

3. Place the bell jar mouth downwards in the mercury—first seeing that there is free communication between the interior of the jar and the external air—and suck up the mercury into the tap; then shut off the tap.

4. Plug the open end of the three-way tap with melted wax.

5. Connect up the horizontal arm of the culture flask with that of the gas receiver by means of the pressure tubing (after removing the cotton-wool plug from the rubber tube), as shown in Fig. 127.

6. Give the three-way tap a half turn to open communication between flask and receiver, and seal *all*

joints by coating with a film of melted wax. When the tap is turned, the mercury in the receiver will naturally fall.

7. Place the entire apparatus in the incubator. (Two hours later, by which time the temperature of the apparatus is that of the incubator, mark the height of the mercury on the receiver.)

8. Examine the apparatus from day to day and mark the level of the mercury in the receiver at intervals of twenty-four hours.

9. When the evolution of gas has ceased, remove

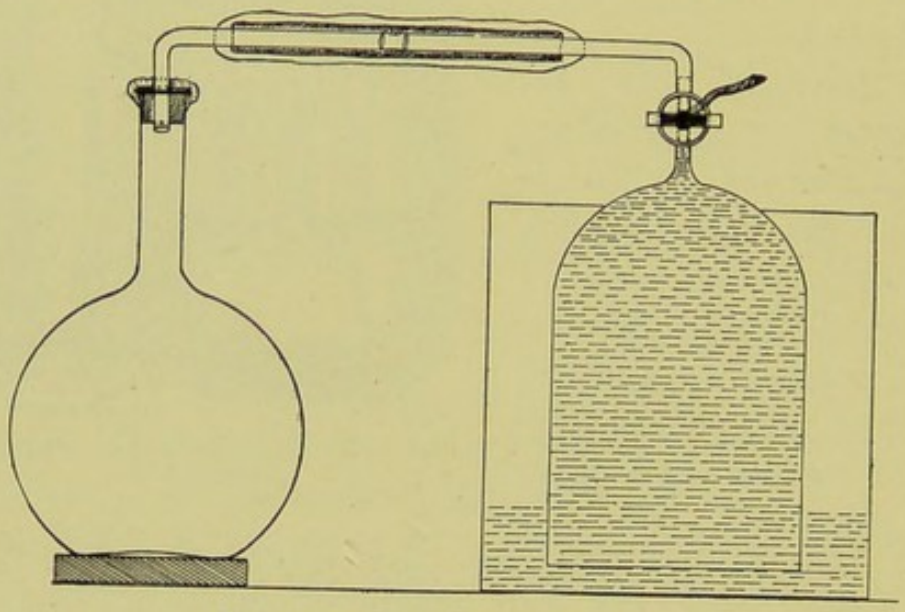


Fig. 127.—Gas-collecting apparatus.

the apparatus from the incubator; clear out the wax from the nozzle of the three-way tap (first adjusting the tap so that no escape of gas shall take place) and connect it with the Orsat.

10. Remove, say, 100 c.c. of gas from the receiver, reverse the tap and force it into the culture flask. Remove 100 c.c. of mixed gases from the culture flask and replace in the receiver.

Repeat these processes three or four times to ensure thorough admixture of the contents of flask and receiver.

11. Now withdraw a sample of the mixed gases into the Orsat and analyse.

In calculating the results be careful to allow for the volume of air contained in the flask at the commencement of the experiment.

For the collection of gases formed under anaerobic conditions a slightly different procedure is adopted:

1. Fix a culture flask (500 c.c. capacity) with a perforated rubber stopper carrying an L-shaped piece of manometer tubing, each arm 5 cm. in length.

2. Prepare a second L-shaped piece of tubing, the short arm 5 cm. and the long arm 20 cm., and connect its short arm to the horizontal arm of the tube in the culture flask by means of a length of pressure tubing, provided with a screw clamp.

3. Fill the culture flask completely with boiling medium and pass the long piece of tubing through the plug of an Erlenmeyer flask (150 c.c. capacity) which contains 100 c.c. of the same medium.

4. Sterilise these coupled flasks by the discontinuous method, in the usual manner.

Immediately the last sterilisation is completed, screw up the clamp on the pressure tubing which connects them, and allow them to cool.

As the fluid cools and contracts it leaves a vacuum in the neck of the flask below the rubber stopper.

5. To inoculate the culture flask, withdraw the long arm of the bent tube from the Erlenmeyer flask and pass it to the bottom of a test-tube containing a young cultivation (in a fluid medium similar to that contained in the culture flask) of the organism it is desired to investigate.

6. Slightly release the clamp on the pressure tubing to allow 4 or 5 c.c. of the culture to enter the flask.

7. Clamp the rubber tube tightly; remove the bent glass tube from the culture tube and plunge it into a

flask containing recently boiled and quickly cooled distilled water.

8. Release the clamp again and wash in the remains of the cultivation until the culture flask and tubing are completely filled with water.

9. Clamp the rubber tubing tightly and take away the long-armed glass tubing.

10. Prepare the gas receiver as in the previous method (in this case, however, the mercury should be warmed slightly) and fill the horizontal arm of the receiver with hot water.

11. Connect up the culture flask with the horizontal arm of the gas receiver.

12. Remove the screw clamp from the rubber tubing, adjust the three-way tap, seal all joints with melted wax, and incubate.

13. Complete the investigation as described for the previous method.

BY PHYSICAL METHODS.

Examine cultivations of the organism with reference to the following points:

Atmosphere:

(a) In the presence of oxygen.
(b) In the absence of oxygen.
(c) In the presence of gases other than oxygen.

Temperature:

(a) Range.
(b) Optimum.
(c) Thermal death-point:

Moist: Vegetative forms.
Spores.
Dry: Vegetative forms.
Spores.

Reaction of medium.

Resistance to lethal agents:

(a) Desiccation.

(b) Light: Diffuse.
Direct.
Primary colours.

(c) Heat.

(d) Chemical antiseptics and disinfectants.

Vitality in artificial cultures.

Agglutination reaction.

I. Atmosphere.—The question as to whether the organism under observation is (a) an obligate aerobe, (b) a facultative anaerobe, or (c) an obligate anaerobe is roughly decided by the appearance of cultivations in the fermentation tubes. Obvious growth in the closed branch as well as in the bulb will indicate that it is a facultative anaerobe; whilst growth only occurring in the bulb or in the closed branch shows that it is an obligate aerobe or anaerobe respectively. This method, however, is not sufficiently accurate for the present purpose, and the examination of an organism with respect to its behaviour in the absence of oxygen is carried out as follows:

Apparatus Required:
Buchner's tubes.
Bulloch's apparatus.
Exhaust pump.
Pyrogallic acid.
Dekanormal caustic soda.
Media Required:
Glucose formate agar.
Glucose formate gelatine.
Glucose formate bouillon.

METHOD.—

1. Prepare four sets of cultivations:

(A) Oblique glucose formate agar and incubate aerobically at 37° C.

Oblique glucose formate gelatine and incubate aerobically at 20° C.

(B) Oblique glucose agar and incubate anaerobically at 37° C.

Oblique glucose formate gelatine. and incubate anaerobically at 20° C.

(C) Oblique glucose formate agar and incubate anaerobically at 37° C.

Glucose formate bouillon and incubate anaerobically at 37° C.

(D) Oblique glucose formate gelatine and incubate anaerobically at 20° C.

Glucose formate bouillon and incubate anaerobically at 20° C.

2. Seal the cultures forming set B in Buchner's tubes (*vide* page 189).

3. Seal the cultures forming sets C and D in Bulloch's apparatus; exhaust the air by means of a vacuum pump, and provide for the absorption of any residual oxygen by the introduction of pyrogallic acid and caustic soda solution (*vide* page 194).

4. Observe the cultivations macroscopically and microscopically at intervals of twenty-four hours until the completion, if necessary, of seven days' incubation.

5. Control these results.

Gases Other than Oxygen.—

Apparatus Required:

Bulloch's apparatus.
Sterile gas filter (*vide* page 43).
Gasometer containing the gas it is desired to test or gas generator for the production of SO_2, N_2O, NO, CO_2, coal gas, etc.

METHOD.—

1. Prepare tube cultivations upon the surface of solid media and deposit them in Bulloch's apparatus.

2. Connect up the inlet tube of the Bulloch's jar with the sterile gas filter, and this again with the delivery tube of the gasometer or gas generator.

3. Open both stop-cocks of the Bulloch's apparatus and pass the gas through until it has completely replaced the air in the bell jar.

4. Incubate under optimum conditions as to temperature.

5. Examine the cultivations at intervals of twenty-four hours, until the completion of seven days.

6. Remove one tube from the interior of the apparatus each day. If no growth is visible, incubate the tube under optimum conditions as to temperature *and* atmosphere, and in this way determine the length of exposure to the action of the gas necessary to kill the organisms under observation.

7. Control these results.

II. Temperature.—

(*A*) *Range.—*

1. Prepare a series of ten tube cultivations, in fluid media, of optimum reaction.

2. Arrange a series of incubators at fixed temperature, varying 5° C. and including temperatures between 5° C. and 50° C.

(Or utilise the water-bath employed in testing the thermal death-point of vegetative forms.)

3. Incubate one tube cultivation of the organism aerobically or anaerobically, as may be necessary, in each incubator, and examine at half-hour intervals for from five to eighteen hours.

4. Note that temperature at which growth is first observed macroscopically.

5. Continue the incubation until the completion of seven days. Note the extremes of temperature at which growth takes place (Range of temperature).

6. Control these results.

(*B*) *Optimum.—*

1. Prepare a second series of ten tube cultivations under similar conditions as to atmosphere and reaction of medium.

2. Incubate in a series of incubators in which the temperature is regulated at intervals of 1° C. for five

degrees on either side of optimum temperature observed in the previous experiment, step 4.

3. Observe again at half-hour intervals and note that temperature at which growth is first visible to the naked eye = Optimum temperature.

(*C*) *Thermal Death-point.*—

Moist—Vegetative Forms:

Apparatus Required:

Water-bath. For the purpose of observing the thermal death-point a special water-bath is necessary. The temperature of this piece of apparatus is controlled by

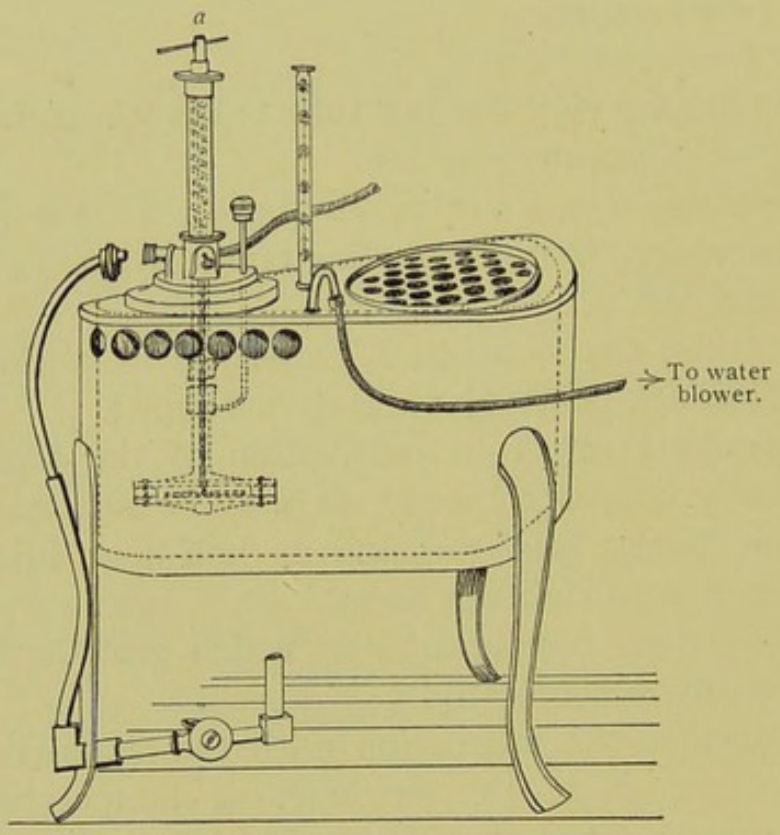


Fig. 128.—Hearson's water-bath.

means of a capsule regulator and can be regulated for intervals of half a degree centigrade through a range of 30°, from 50° C. to 80° C. by means of a spring, actuated by the handle *a*, which increases the pressure in the interior of the capsule. A hole is provided for the reception of the nozzle of a blast pump, so that a current of air may be blown through the water whilst the bath is in use, and thus ensure a uniform temperature of its contents.

Sterile capsules.
Flask containing 250 c.c. sterile normal saline solution.
Case of sterile pipettes, 10 c.c. (in tenths of a cubic centimetre).
Special loop.
Test-tubes, 18 by 1.5 cm., of thin German glass.

METHOD.—

1. Prepare several tube cultivations on solid media of optimum reaction and incubate for forty-eight hours under optimum conditions as to temperature and atmosphere.

2. Examine preparations from the cultivation microscopically to determine the absence of spores.

3. Pipette 5 c.c. salt solution into each of twelve capsules.

4. Suspend three loopfuls of the growth from the surface of the medium (using a carefully made platinum loop especially reserved for this purpose) in the normal saline solution in each capsule.

5. Transfer each suspension to a sterile test-tube and number consecutively from 1 to 12.

6. Adjust the first tube in the water-bath, regulated at 40° C., by means of two rubber rings around the tube, one above and the other below the perforated top of the bath, so that the upper level of the fluid in the tube is about 4 cm. below the surface of the water in the bath, and the bottom of the tube is a similar distance above the bottom of the bath.

7. Arrange a control test-tube containing 5 c.c. water under similar conditions. Plug the tube with cotton-wool and pass a thermometer through the plug so that its bulb is immersed in the water.

8. Close the unoccupied perforations in the lid of the water-bath by means of glass balls.

9. Watch the thermometer in the test-tube until it records a temperature of 40° C. Note the time. Ten minutes later remove the tube containing the sus-

pension, and cool rapidly by immersing its lower end in a stream of running water.

10. Pour three gelatine (or agar) plates containing respectively 0.2, 0.3, and 0.5 c.c. of the suspension, and incubate.

11. Pipette the remaining 4 c.c. of the suspension into a culture flask containing 250 c.c. of nutrient bouillon, and incubate.

12. Observe these cultivations from day to day. "No growth" must not be recorded as final until after the completion of seven days' incubation.

13. Extend these observations to the remaining tubes of the series, but varying the conditions so that each tube is exposed to a temperature 2° C. higher than the immediately preceding one—*i. e.*, 42° C., 44° C., 46° C., and so on.

14. Note that temperature, after exposure to which no growth takes place up to the end of seven days' incubation, = the thermal death-point.

15. If greater accuracy is desired, a second series of tubes may be prepared and exposed for ten minutes to fixed temperatures varying only 0.5° C., through a range of 5° C. on either side of the previously observed death-point.

Moist—Spores: The thermal death-point in the case of spores is that time exposure to a temperature of 100° C. necessary to effect the death of all the spores present in a suspension.

It is determined in the following manner

Apparatus Required:

Steam can be fitted with a delivery tube and a large bore safety-valve tube.

Water-bath at 100° C.

Erlenmeyer flask, 500 c.c. capacity, containing 140 c.c. sterile normal saline solution and fitted with rubber stopper perforated with four holes.

The rubber stopper is fitted as follows:

(a) Thermometer to 120° C., its bulb immersed in the normal saline.

(b) Straight entry tube, reaching to the bottom of the flask, the upper end plugged with cotton-wool.

(c) Bent syphon tube with pipette, nozzle attached by means of rubber tubing and fitted with pinch-cock.

The nozzle is protected from accidental contamination by passing it through the cotton-wool plug of a small test-tube.

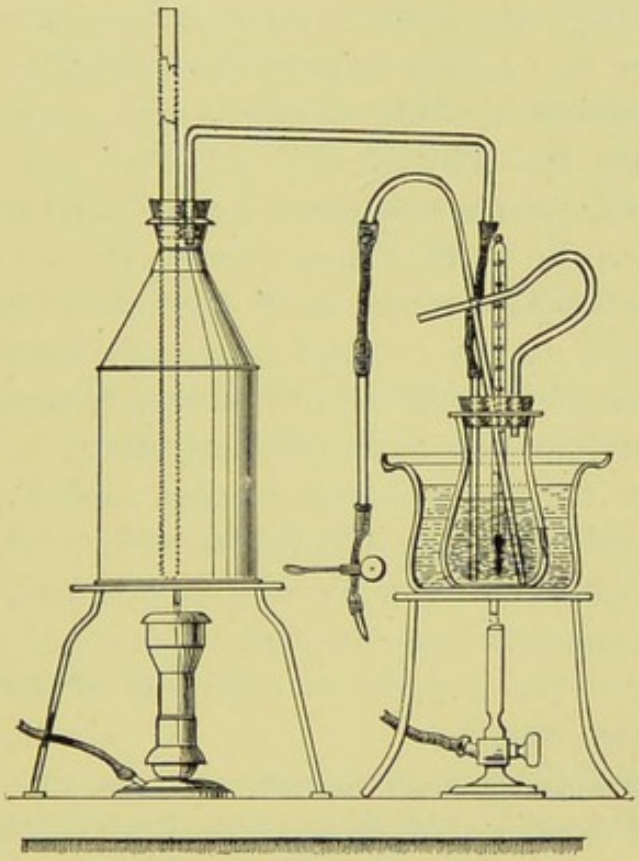


Fig. 129.—Apparatus arranged for the determination of the death-point of spores.

(d) A sickle-shaped piece of glass tubing passing just through the stopper, plugged with cotton-wool, to act as a vent for the steam.

Sterile plates.
Sterile pipettes.
Sterile test-tubes graduated to 5 c.c.

Media Required:

Gelatine or agar.
Culture flasks containing 200 c.c. nutrient bouillon.

METHOD.—

1. Prepare twelve tube cultivations upon the surface (or two cultures in large flat culture bottles—*vide* page 19) of nutrient agar and incubate under the optimum conditions (previously determined), for the formation of spores.

Examine preparations from the cultures microscopically to determine the presence of spores.

2. Pipette 5 c.c. sterile normal saline into each culture tube or 30 c.c. into each bottle and by means of a sterile platinum spatula emulsify the entire surface growth with the solution.

3. Add the 60 c.c. emulsion to 140 c.c. normal saline contained in the fitted Erlenmeyer flask.

4. Place the flask in the water-bath of boiling water.

5. Connect up the straight tube, after removing the cotton-wool plug, with the delivery tube of the steam can; remove the plug from the vent tube.

6. When the thermometer reaches 100° C., *syphon* off 5 c.c. of the suspension into the sterile graduated test-tube and pour plates and prepare flask cultures as in the previous experiments.

7. Repeat this process at intervals of twenty-five minutes' steaming.

8. Control these experiments, but in this instance syphon off portions of the suspension at intervals of one-half or one minute during the five or ten minutes preceding the previously determined death-point.

Thermal Death-point.—

Dry—Vegetative Forms:

Apparatus Required:

Hot-air oven, provided with thermo-regulator.
Sterile cover-slips.
Flask containing 250 c.c. sterile normal saline solution.
Case of sterile pipettes, 10 c.c. (in tenths of a cubic centimetre).
Case of sterile capsules.
Crucible tongs.

METHOD.—

1. Prepare an emulsion with three loopfuls from an optimum cultivation in 5 c.c. normal saline in a sterile capsule and examine microscopically to determine the absence of spore forms.

2. Make twelve cover-slip films on sterile cover-slips; place each in a sterile capsule to dry.

3. Expose each capsule in turn in the hot-air oven for ten minutes to a different fixed temperature, varying 5° C. between 60° C. and 120° C.

4. Remove each capsule from the oven with crucible tongs immediately the ten minutes are completed; remove the cover-glass from its interior with a sterile pair of forceps.

5. Deposit the film in a flask containing 200 c.c. nutrient bouillon.

6. Prepare subcultivations from such flasks as show evidence of growth, to determine that no contamination has taken place.

7. Control the result of these experiments.

Dry—Spores:

Apparatus Required:

As for vegetative forms.

METHOD.—

1. Prepare an oblique agar tube cultivation and incubate under optimum conditions as to spore formations.

2. Pipette 5 c.c. sterile normal saline into the culture tube and emulsify the entire surface growth in it. Examine microscopically to determine the presence of spores in large numbers.

3. Spread thin even films on twelve sterile cover-slips and place each cover-slip in a separate sterile capsule.

4. Expose each capsule in turn for ten minutes to a different fixed temperature, varying 5° C., between 100° C. and 160° C.

5. Complete the examination as for vegetative forms.

III. Reaction of Medium.—

(*A*) *Range.—*

1. Prepare a bouillon culture of the organism and incubate, under optimum conditions as to temperature and atmosphere, for twenty-four hours.

2. Pipette 0.1 c.c. of the cultivation into a sterile capsule; add 9.9 c.c. sterile bouillon and mix thoroughly.

3. Prepare a series of tubes of nutrient bouillon of varying reactions, from +25 to —30 (*vide* page 133), viz.: +25, +20, +15, +10, +5, neutral, —5, —10, —15, —20, —25, —30.

4. Inoculate each of the bouillon tubes with 0.1 c.c. of the diluted cultivation and incubate under optimum conditions.

5. Observe the cultures at half-hourly intervals from the third to the twelfth hours. Note the reaction of the tube or tubes in which growth is first visible macroscopically (probably optimum reaction).

6. Continue the incubation until the completion of forty-eight hours. Note the extremes of acidity and alkalinity in which macroscopical growth has developed (Range of reaction).

7. Control the result of these observations.

(*B*) *Optimum Reaction.*—The optimum reaction has already been roughly determined whilst observing the range. It can be fixed within narrower limits by inoculating in a similar manner a series of tubes of bouillon which have a smaller variation in reaction than those previously employed (say, 1 instead of 5) for five points on either side of the previously observed optimum. For example, optimum reaction in the set of experiments to determine the range was +10. Now plant tubes having reactions of +15, +14, +13, +12, +11, +10, +9, +8, +7, +6, +5, and observe as before.

IV. Resistance to Lethal Agents.—

(*A*) *Desiccation.*—

Apparatus Required:

Müller's desiccator. This consists of a bell glass fitted with an exhaust tube and stop-cock (*d*), which can be secured to a plate-glass base (*c*) by means of wax or grease. It contains a cylindrical vessel of porous clay (*a*) into which pure sulphuric acid is poured whilst the material to be dried is placed within its walls on a glass shelf (*b*). The air is exhausted from the interior and the acid rapidly converts the clay vessel into a large absorbing surface.

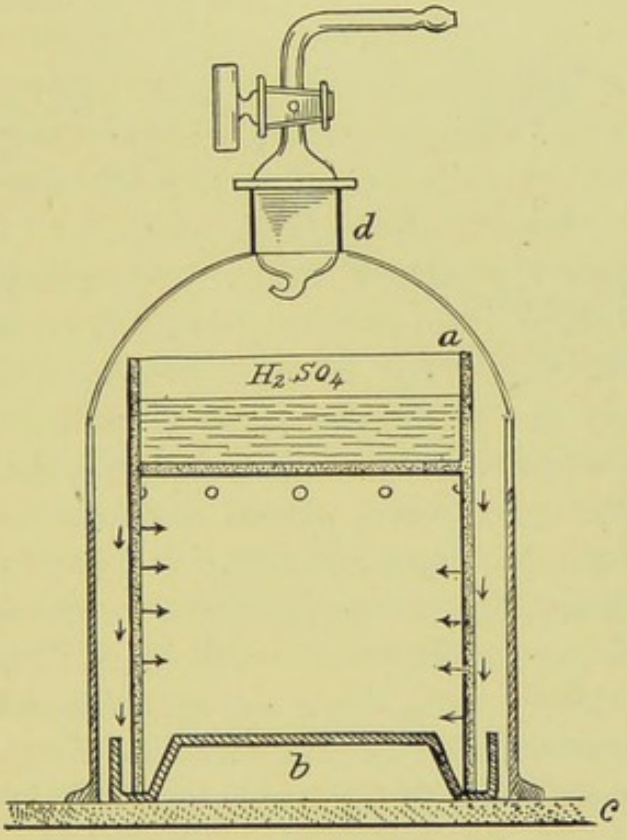


Fig. 130.—Müller's desiccator.

Exhaust pump.
Pure concentrated sulphuric acid.
Sterile cover-slips.
Sterile forceps.
Culture flask containing 200 c.c. nutrient bouillon.
Sterile ventilated Petri dish. This is prepared by bending three short pieces of aluminium wire into V shape and hanging these on the edge of the lower dish and resting the lid upon them (Fig. 131).

METHOD.—

1. Prepare a surface cultivation on nutrient agar in a culture bottle and incubate under optimum conditions for forty-eight hours.

2. Examine preparations from the cultivation, microscopically, to determine the absence of spores.

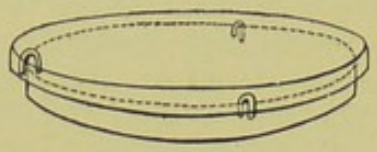


Fig. 131.—Petri dish for drying cultivations.

3. Pipette 5 c.c. sterile normal saline solution into the flask and suspend the entire growth in it.

4. Spread the suspension in thin, even films on sterile cover-slips and deposit inside sterile "plates" to dry.

5. As soon as dry, transfer the cover-slip films to the ventilated Petri dish by means of sterile forceps.

6. Place the Petri dish inside the Müller's desiccator; fill the upper chamber with pure sulphuric acid, cover with the bell jar, and exhaust the air from its interior.

7. At intervals of five hours admit air to the apparatus, remove one of the cover-slip films from the Petri dish, and transfer it to the interior of a culture flask, with every precaution against contamination. Reseal the desiccator and exhaust.

8. Incubate the culture flask under optimum conditions until the completion of seven days, if necessary.

9. Pour plates from those culture flasks which grow, to determine the absence of contamination.

10. Repeat these observations at hourly intervals for the five hours preceding and succeeding the death time, as determined in the first set of experiments.

(*B*) *Light.*—

(a) Diffuse Daylight:

1. Prepare a tube cultivation in nutrient bouillon, and incubate under optimum conditions, for forty-eight hours.

2. Pour twenty plate cultivations, ten of nutrient

gelatine and ten of nutrient agar, each containing 0.1 c.c. of the bouillon culture.

3. Place one agar plate and one gelatine plate into the hot and cold incubators, respectively, as *controls*.

4. Fasten a piece of black paper, cut the shape of a cross or star, on the centre of the cover of each of the remaining plates (Fig. 132).

5. Expose these plates to the action of diffuse daylight (not direct sunlight) in the laboratory for one, two, three, four, five, six, eight, ten, twelve hours.

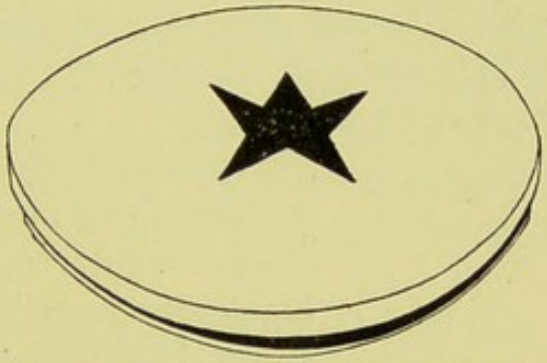


Fig. 132.—Plate with star for testing effect of light.

6. After exposure to light, incubate under optimum conditions.

7. Examine the plate cultivations after twenty-four and forty-eight hours' incubation, and compare with the two controls. Record results. If growth is absent from that portion of the plate unprotected by the black paper, continue the incubation and daily observation until the end of seven days.

8. Control the results.

(b) Direct Sunlight:

1. Prepare plate cultivations precisely as in the former experiments and place the two controls in the incubators.

2. Arrange the remaining plates upon a platform in the direct rays of the sun.

3. On the top of each plate stand a small glass dish 14 cm. in diameter and 5 cm. deep.

4. Fill a solution of potash alum (2 per cent. in distilled water) into each dish to the depth of 2 cm. to absorb the heat of the sun's rays and so eliminate possible effects of temperature on the cultivations.

5. After exposures for periods similar to those employed in the preceding experiment, incubate and complete the observation as above.

(c) Primary Colours: Each colour—violet, blue, green, red, and yellow—must be tested separately.

1. Prepare plate cultivations, as in the previous "light" experiments, and incubate controls.

2. Fasten a strip of black paper, 3 cm. wide, across one diameter of the cover of each plate.

3. Coat the remainder of the surface of the cover with a film of pure photographic collodion which contains 2 per cent. of either of the following aniline dyes, as may be necessary:

Chrysoidin (for red).
Aurantia (for orange).
Naples yellow (for yellow).
Malachite green (for green).
Eosin, bluish (for blue).
Methyl violet (for violet).

4. Expose the plates, thus prepared, to bright daylight (but not direct sunlight) for varying periods, and complete the observations as in the preceding experiments.

5. Control the results.

(*C*) *Heat.*—(*Vide* Thermal Death-point, page 242.)

(*D*) *Antiseptics and Disinfectants.*—(*Vide* Testing Germicides, page 359.) Testing the organism under observation against, for example,

Bichloride of mercury;
Formaldehyde;
Carbolic acid;

noting (*a*) strength of solution; (*b*) duration of exposure necessary to produce death.

The Agglutination Reaction.—This test, which is variously known as the agglutination reaction, clumping reaction, or Gruber's reaction, depends upon the fact that the blood-serum of an animal immunised against

a certain micro-organism possesses the power of agglutinating (or collecting together in clumps and masses) watery suspensions of that particular microbe. It was first applied by Durham and Gruber in the identification of races of cholera vibrios, and was afterwards extended by various workers to other species of bacteria, such as B. typhosus, B. pestis, M. melitensis, etc.

The converse of the test—viz., the diagnosis of disease by the determination of the particular pathogenic organism that is agglutinated by the blood-serum of the patient—is often termed Widal's reaction, and is extensively used in the diagnosis of enteric fever, when the serum from a suspected case of typhoid is tested against a bouillon cultivation of an authentic Bacillus typhosus.

It is now generally agreed that the reaction is unreliable unless performed under certain conditions.

1. As to the period of time the suspension of the organism is in contact with specific serum. This must not exceed thirty minutes.

2. As to the strength of the solution of specific serum employed in the test. This must not exceed 5 per cent.

The method of employing the test and the preparation of the serum solution are best considered separately, and the test itself illustrated by an example such as the confirmation of the identity of a bacillus provisionally regarded as the B. typhosus.

(*A*) *Preparation.*—

Collection of the Specific Serum:

Apparatus Required:
Razor.
Liquid soap.
Cotton-wool.
Two per cent. lysol solution in drop bottle.
Absolute alcohol in drop bottle.
Hare-lip pin or pointed scalpel.
Blood pipette (*vide* page 22).

METHOD.—

1. Select a rabbit which has been immunised to the B. typhi abdominalis, and have it firmly held by an assistant.

2. Shave the dorsal surface of the ear, over the posterior auricular vein.

NOTE.—The serum may also be obtained from the lobe of the ear of a patient suffering or convalescent from enteric fever, by carrying out the succeeding steps (see Fig. 133).

3. Sterilise the skin by washing with lysol.

The lysol should be applied with sterile cotton-wool

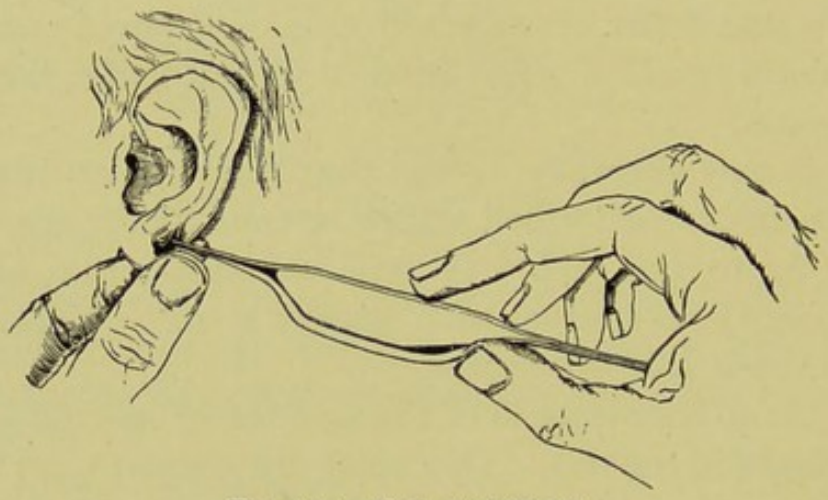


Fig. 133.—Collecting blood.

and the ear vigourously rubbed, not only to remove superficial scales of epithelium, but also to render the ear hyperæmic and the vein prominent.

4. Remove the lysol with absolute alcohol.

5. Dry the sterilised area of skin with sterile cotton-wool.

6. Puncture the vein with the sterile hare-lip pin and collect the issuing blood in the blood pipette, thus:

Hold one of the narrow tube-ends in contact with the blood and depress the other end. The blood will run into the pipette by gravity. When the tube is full to the shoulder, remove the pipette, place the clean end

to the lips, and aspirate gently, so that the blood flows into the barrel of the pipette.

Hold the pipette horizontally, and seal the ends in a Bunsen flame.

Rest the pipette in the horizontal position, by its ends on the rim of a tumbler or beaker, so that its barrel is suspended, and allow the blood to coagulate (Fig. 134, *a*). This will probably take about twenty to thirty minutes.

Place the pipette in the vertical position, clean end downwards, in a beaker or wire stand, and set it in

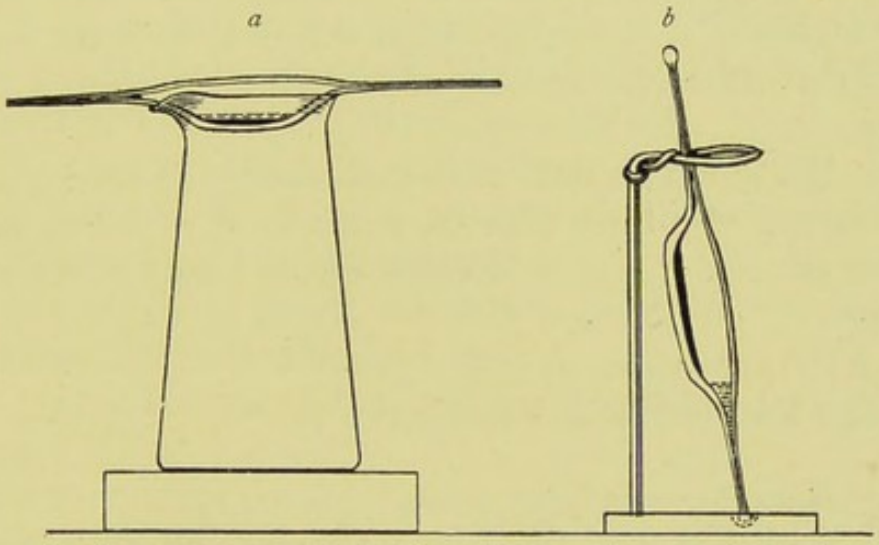


Fig. 134.—Collecting serum: *a*, formation of clot; *b*, separation of serum.

the ice-chest for an hour or so, for the clear serum to separate and collect in the clean end of the pipette (Fig. 134, *b*).

Dilution of the Specific Serum:

Apparatus Required:

Sterile graduated capillary pipettes (to contain 10 c.mm.).
Sterile graduated capillary pipettes (to contain 90 c.mm.).
Small sterile test-tubes (5 cm. by 0.5 cm.).
Tube of nutrient bouillon.
Pipette of specific serum.
Three-square file.
Glass capsule, nearly full of dry silver sand.
Grease pencil.

METHOD.—

1. Take three sterile test-tubes and number them 1, 2, and 3.

2. Pipette 90 c.mm. sterile bouillon into each tube, and stand it upright in the sand in the capsule.

3. Make a file scratch on the blood pipette above the upper level of the clear serum, and snap off the narrow tube containing the serum.

4. Remove 10 c.mm. of the serum from the blood pipette tube, and mix it thoroughly with the bouillon in tube No. 1; = specific serum solution, 10 per cent.

5. Remove 10 c.mm. of the solution from tube No. 1 by means of a fresh pipette, and mix it with the contents of tube No. 2; = specific serum solution, 1 per cent.

6. Remove 10 c.mm. of the solution from tube No. 2 by means of a fresh pipette, and mix it with the contents of tube No. 3; = specific serum solution, 0.1 per cent.

(*B*) *Application.*—

The Microscopical Reaction:

Apparatus Required:

Five hanging-drop slides (or preferably two slides, with two cells mounted side by side on each (Fig. 47, *a*), and one slide with one cell only).
Vaseline.
Cover-slips.
Platinum loop.
Grease pencil.
Eighteen- to twenty-four-hour-old bouillon cultivation of the organism to be tested (Bacillus typhi abdominalis?).
Pipette end with the remainder of the undiluted serum.
Tubes containing the three solutions of the specific serum, 10, 1, and 0.1 per cent. respectively.

METHOD.—

1. Make five hanging-drop preparations, thus:

(a) One loopful of bouillon cultivation + one loopful sterile bouillon; label "Control."

(b) One loopful culture + one loopful undiluted serum; label 50 per cent.

Mount these two cover-slips on a double-celled slide.

(c) One loopful bouillon culture + one loopful 10 per cent. serum; label 5 per cent.

Mount this on single-cell slide.

(d) One loopful bouillon culture + one loopful 1 per cent. serum; label 0.5 per cent.

(e) One loopful bouillon culture + one loopful 0.1 per cent. serum; label 0.05 per cent.

Mount these two cover-slips on a double-celled slide.

2. Note the time: Examine the control to determine that the bacilli are motile and uniformly scattered over the field—not collected into masses.

3. Next examine the 50 per cent. preparation.

If the test is giving a positive reaction, the bacilli *will* be collected in large clumps.

If the test is giving a negative reaction, the bacilli *may* be collected in large clumps.

4. Observe the 5 per cent. preparation microscopically.

If the bacilli are aggregated into clumps, positive reaction.

If the bacilli are *not* aggregated into clumps, observe until thirty minutes from the time of preparation before recording a negative reaction.

5. Examine the 0.5 and 0.05 per cent. preparations. These may or may not show agglutination when the result of the examination of the 5 per cent. preparation is positive, according to the potency of the specific serum; and by dilutions such as these a quantitative comparison of the valency of specific sera may be obtained.

NOTE.—The graduated pipettes supplied with Thoma's hæmatocytometer (intended for the collection of the specimen of blood required for the enumeration

of leucocytes), giving a dilution of 1 in 10,—*i. e.*, 10 per cent.,—may be substituted for the graduated capillary pipettes referred to above, if the vessel in which the serum has been separated is of sufficiently large diameter to permit of their use.

A handy, though somewhat crude, method of applying this microscopical test is carried out as follows:

Apparatus Required:

Pipette containing immune serum.
Eighteen- to twenty-four-hour-old broth cultivation of the organism to be tested.
Tube of sterile broth (or sterile water).
Cover-slips.
Platinum loop.
Hanging-drop slides.
Vaseline.
Grease pencil.

METHOD.—

1. Flame a clean cover-slip and rest it on a piece of blotting paper.

2. By means of a sterile platinum loop place nine as nearly as possible equal loopfuls of sterile broth (or sterile water) on the surface of the cover-slip.

3. Sterilise the loop, fill it once with the serum to be tested, and mix thoroughly with the nine drops of diluent. This gives approximately a 10 per cent. solution of serum.

4. Flame a second clean cover-slip and place it by the side of the first.

5. Sterilise the loop, charge it with the serum solution, and deposit a drop on the surface of the second cover-slip.

6. Sterilise the loop, and add a loopful of the broth cultivation to the drop of diluted serum, and mix thoroughly.

7. Mount the cover-slip as a hanging drop, label 5 per cent., and examine microscopically.

Higher dilutions can be prepared in a similar manner.

The Macroscopical Reaction:

Apparatus Required:

Sterile graduated capillary pipettes to contain 90 c.mm.
Eighteen- to twenty-four-hour-old bouillon cultivation of the organism to be tested.
Three test-tubes containing the 10, 1, and 0.1 per cent. solutions of specific serum (about 90 c.mm. remaining in each).
Sedimentation tubes (*vide* page 23).

METHOD.—

1. Pipette 90 c.mm. of the bouillon culture into each of the tubes containing the diluted serum.

2. Fill a sedimentation tube (by aspirating) from the contents of each tube. Seal off the lower ends of the sedimentation tubes in the Bunsen flame.

3. Label each tube with the dilution of serum that it contains—viz., 5, 0.5, and 0.05 per cent.

4. Place the pipettes in a vertical position, in a beaker, in the incubator at 37° C., for one or two hours.

5. Observe the granular precipitate which is thrown down when the reaction is positive, and the uniform turbidity of the negative reaction.

PATHOGENESIS.

1. *Living Bacteria.*—

(a) Psychrophilic Bacteria: When the organism will only grow at or below 18° to 20° C.,

1. Prepare cultivations in nutrient broth and incubate under optimum conditions.

2. After seven days' incubation inject that amount of the culture corresponding to 1 per cent. of the body-weight of a selected frog, into its dorsal lymph sac.

3. Observe until death takes place, or, in the event of a negative result, until the completion of twenty-eight days.

4. If death occurs, make a careful post-mortem examination (*vide* page 287).

(b) Mesophilic Bacteria: When the organism grows at 35° to 37° C.,

1. Prepare cultivations in nutrient broth and incubate under optimum conditions for forty-eight hours.

2. Inoculate a selected white mouse, subcutaneously at the root of the tail, with an amount of cultivation equivalent to 1 per cent. of its body-weight.

3. Inoculate a second mouse intraperitoneally with a similar dose.

4. Observe carefully until death occurs, or until the lapse of twenty-eight days.

5. If the inoculated animals succumb, make complete post-mortem examination.

If death follows shortly after the injection of cultivations of bacteria, the inoculation experiments should be repeated two or three times. Then, if the organism under observation invariably exhibits pathogenic effects, steps should be taken to ascertain, if possible, the minimal lethal dose (as described on page 269) of the growth upon solid media for the frog or white mouse respectively. Other experimental animals—*e. g.*, the white rat, guinea-pig, and rabbit—should next be tested in a similar manner.

2. *Toxins.*—Prepare cultivations of the organism under observation in glucose formate broth, and incubate for fourteen days under optimum conditions.

(a) Intracellular or Insoluble Toxins:

1. Heat the fluid culture in a water-bath at 60° C. for twenty minutes. (The resulting sterile, turbid fluid is often spoken of as "killed" culture.)

2. Inject subcutaneously that amount of the cultivation corresponding to 1 per cent. of the body-weight of the selected animal, usually one of the small rodents.

3. Inoculate a tube of sterile bouillon with a similar quantity, and inoculate under optimum conditions. This "control" then serves to demonstrate the freedom of the toxin from living bacteria.

4. Observe during life or until the completion of twenty-eight days, and in the event of death occurring during that period, make a complete post-mortem examination.

5. Repeat the experiment at least once. In the event of a positive result estimate the minimal lethal dose of "killed" culture for each of the species of animals experimented upon.

(b) Extracellular or Soluble Toxins:

1. Filter the cultivation through a porcelain filter candle (Berkfeld) into a sterile filter flask, arranging the apparatus as in the accompanying figure (Fig. 135).

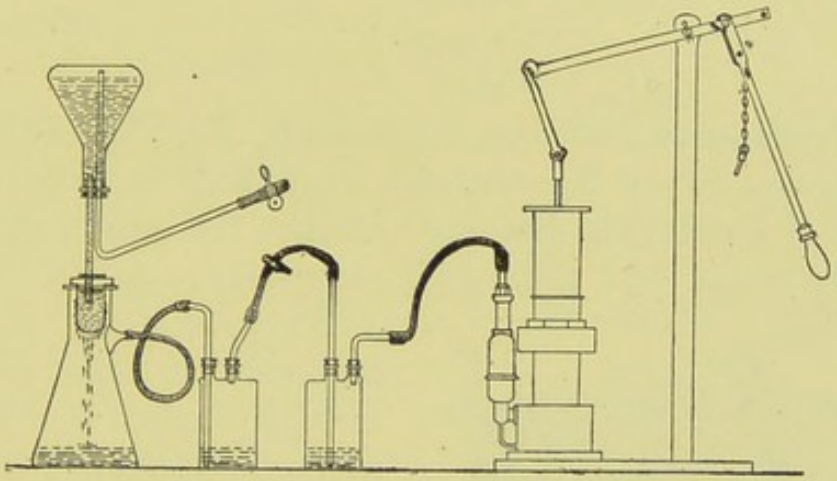


Fig. 135.—Apparatus arranged for toxin filtration.

2. Inoculate mice, rats, guinea-pigs, and rabbits subcutaneously with that quantity of toxin corresponding to 1 per cent. of the body-weight of each respectively, and observe, if necessary, until the completion of one month.

3. Inoculate a "control" tube of bouillon with a similar quantity and incubate.

4. In the event of a fatal termination make complete and careful post-mortem examinations.

5. Repeat the experiments and, if the results are positive, ascertain the minimal lethal dose of toxin for each of the susceptible animals.

XV. EXPERIMENTAL INOCULATION OF ANIMALS.

The animals generally employed in the study of the pathogenic properties of the various micro-organisms are:

Mouse.
Rat.
Guinea-pig.
Rabbit.
Pigeon.
Fowl.

Preparation.—Before inoculation, the experimental animals should be carefully examined, to avoid the risk of employing diseased animals; the weight should be recorded and the rectal temperature taken.

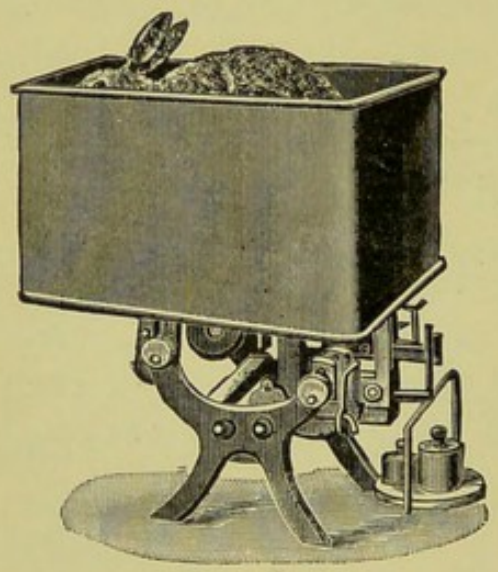


Fig. 136.—Rabbit scales.

Weighing.—The larger animals are most conveniently weighed in a decimal scale provided with a metal cage for their reception instead of the ordinary pan (Fig. 136). Mice and rats are weighed in a modification of the letter balance, weighing to 250 grammes, which has a conical wire cage substituted for its pan (Fig. 137).

The weight of inoculated animals should be observed and recorded each day, at precisely the same hour, during the entire period of observation, preferably before the morning feeding.

Temperature.—To take the rectal temperature of any of the laboratory animals, the animal should be firmly held by an assistant and the bulb of an ordinary clinical thermometer, well greased with vaseline, introduced just within the sphincter ani. Allow it to remain in this position for a few seconds, and then push it on gently and steadily until the entire bulb and part of the stem, as far as the constriction, have passed into the rectum. Three to five minutes later, the time varying, of course, with the sensibility of the thermometer used, withdraw the instrument and take the reading.

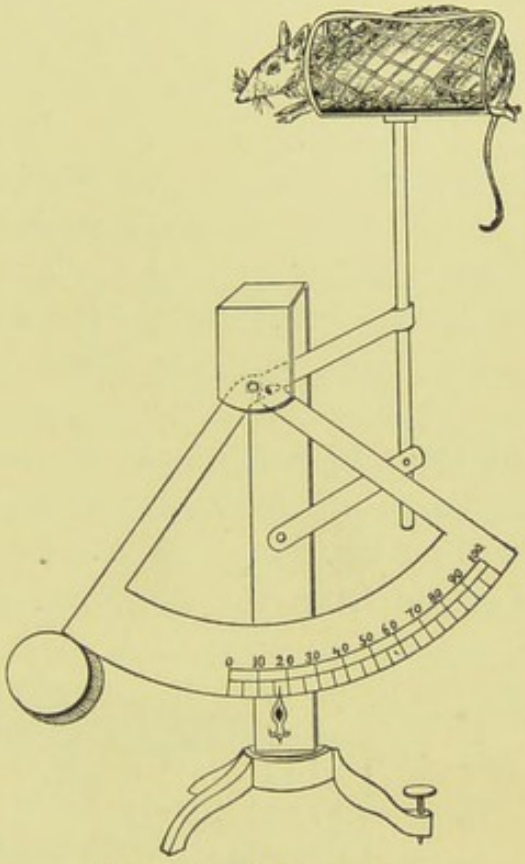


Fig. 137.—Mouse scales.

Daily, if not more frequently, observations should be made of the temperature of inoculated animals during the entire period they are under observation.

Cages.—During the period which elapses between inoculation and death, or complete recovery, the experimental animals must be kept in suitable receptacles that can easily be kept clean and which can be readily disinfected.

The *mouse* is usually stored in a glass jar (Fig. 138) 11 cm. high and 11 cm. in diameter, closed by a wire gauze top which is weighted with lead or fastened to the mouth of the jar by a bayonet catch. A matter of great convenience is a small oblong label 5 cm. by

2.5 cm., sand-blasted on the side of the cylinder, as marks made upon this with an ordinary lead pencil show up well and only require the use of a damp cloth to remove them (Fig. 138).

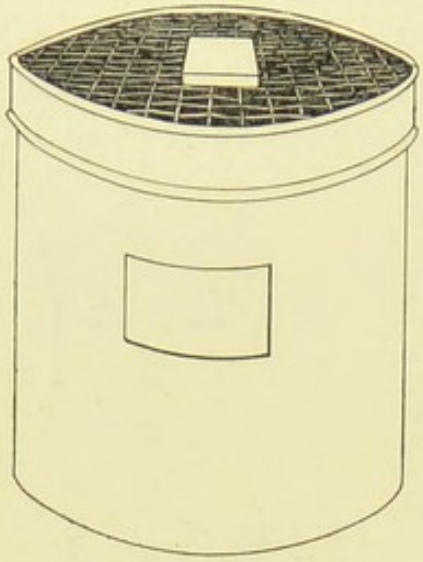


Fig. 138.—Mouse jar.

The *rat* is kept under observation in a glass jar similar, but larger, to that used for the mouse.

These jars are sterilised after use either by chemical reagents or by autoclaving.

The *rabbit* and the *guinea-pig* are confined in cages of suitable size, made entirely of metal (Fig. 139). The sides and top and bottom are of woven wire work; beneath the cage is a movable metal tray filled with sawdust, for the reception of the excreta. The cage as a whole is raised from the ground on short legs. The sides,

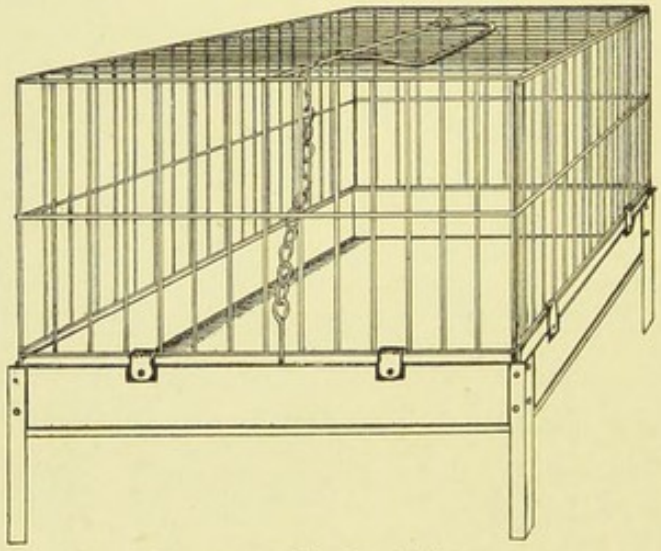


Fig. 139.—Metal rabbit cage.

etc., are generally hinged so that the cage packs up flat, for convenience of storing and also of sterilising.

The ordinary rat cage, a rectangular wire-work

box, 30 cm. from front to back, 20 cm. wide, and 14 cm. high, makes an excellent cage for guinea-pigs if fitted with a shallow zinc tray, 35 by 24 cm., for it to stand upon.

These cages are sterilised after use either by autoclaving or spraying with formalin.

The tray which receives the dejecta should be cleaned out and supplied with fresh sawdust each day, and the soiled sawdust, remains of food, etc., should be cremated.

As **animal inoculation** is purely a surgical operation, the necessary instruments will be similar to those employed by the surgeon, and, like them, must be sterile. In the performance of the inoculation strict attention must be paid to asepsis and suitable precautions adopted to guard against accidental contamination of the material to be introduced into the animal. In addition, the hands of the operator should be carefully disinfected.

The list of apparatus used in animal inoculations given below comprises practically everything needed for any inoculation. Needless to remark, all the apparatus will never be required for any one inoculation.

Apparatus Required for Animal Inoculation:

1. Water steriliser (*vide* page 38). It is also convenient to have a second water steriliser, similar but smaller (23 by 7 by 5 cm.), for the sterilisation of the syringes.

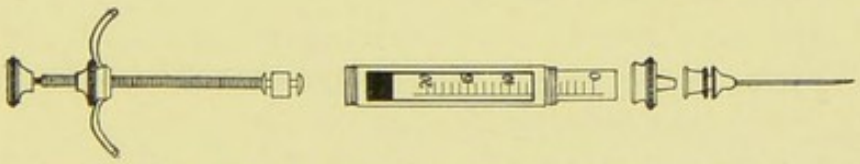


Fig. 140.—Hypodermic syringe with finger rests.

2. Injecting syringe. The best form is one of the ordinary hypodermic pattern, fitted with finger rests, but with the leather washers and the packing of the piston replaced by those made of asbestos (Fig. 140). The instrument must be easily taken to pieces, and

spare parts should be kept on hand to replace accidental breakage or loss. A good supply of needles must be kept on hand, both sharp-pointed and with blunt ends. To sterilise the syringe, fill it with water, loosen the packing of the piston and all the screw joints, place it in the steriliser and boil for at least five minutes. Disinfect the syringe *after use*, in a similar manner. The needles, which are exceedingly apt to rust after being boiled, should be stored in a pot of absolute alcohol when not in use.

3. Surgical instruments, such as
   Scissors, probe and sharp-pointed.
   Dissecting forceps of various patterns.
   Pressure forceps.
   Aneurism needles, sharp and blunt.
   Scalpels, Keratomes, Trephines, } with metal handles.
   Surgical needles.
   Needle holder.
   Sterilise these before use by boiling, and disinfect them *after use* by the same means. Wipe perfectly dry immediately the disinfection is completed.
4. Anæsthetic.
   (*a*) General: The safest general anæsthetic for animals is an A. C. E. mixture, containing by volume alcohol 1 part, chloroform 2 parts, ether 6 parts, and should be administered on a "cone" formed by twisting up one corner of a towel and placing a wad of cotton-wool inside it.
   (*b*) Local:
   Cocaine hydrochloride, 2 per cent. solution.
   Eucaine, 2 per cent. solution.
5. Sterile capsules of various sizes.
6. Cases of sterile pipettes { 10 c.c. (in tenths of a cubic centimetre). 1 c.c. (in tenths of a cubic centimetre).
7. Flasks (75 c.c.) containing sterilised normal saline solution (or sterile bouillon).
8. Sterilised cotton-wool. Cotton-wool is packed loosely in a copper cylinder similar to that used for storing capsules, and sterilised in the hot-air oven.
9. Sterilised gauze. Gauze is sterilised in the same way as cotton-wool.
10. Sterilised silk and catgut for sutures. These are sterilised, as required, by boiling for some ten minutes in the water steriliser.

11. Flexible collodion (or compound tincture of benzoin).
12. Grease pencil.
13. Tie-on celluloid labels, to affix to the cages.
14. Razor.
15. Small pot of warm water.
16. Liquid soap. Liquid soap is prepared as follows: Measure out 100 grammes of soft soap and add to 500 c.c. of 2 per cent. lysol solution in a large glass beaker; dissolve by heating in a water-bath at about 90° C. Bottle and label "Liquid Soap."

Material Utilised for Inoculation.—The material inoculated may be either—

1. Cultures of bacteria—grown in fluid media or on solid media.

2. Metabolic products of bacterial activity—*e. g.*, toxins in solution.

3. Pathological products (fluid secretions and excretions, solid tissues).

The Preparation of the Inoculum.—

(*a*) *Cultivations in Fluid Media.*—

1. Flame the plug of the culture tube.

2. Remove the plug and flame the mouth of the tube.

3. Slightly raise the lid of a sterile capsule, insert the mouth of the culture tube into the aperture and pour some of the cultivation into the capsule.

4. Remove the mouth of the culture tube from the capsule, replace the lid of the latter, flame the mouth of the tube, and replug.

5. Remove the syringe from the steriliser, squirt out the water from its interior, and allow to cool.

6. Raise the lid of the capsule sufficiently to admit the needle of the syringe and draw the required amount of the cultivation into the barrel of the syringe.

(Or, remove a definite measured quantity of the cultivation directly from the tube or flask by means of a sterile graduated pipette, discharge the measured amount into a sterile capsule, and fill into the syringe.)

If it is necessary to introduce a large bulk of fluid into the animal, the cultivation should be transferred with aseptic precautions, to a sterile separatory funnel, preferably of the shape shown in figure 141, and graduated if necessary. This is supported on a retort stand and raised sufficiently above the level of the animal to be injected, so as to secure a good "fall." A long piece of sterile rubber tubing, fitted with an injection

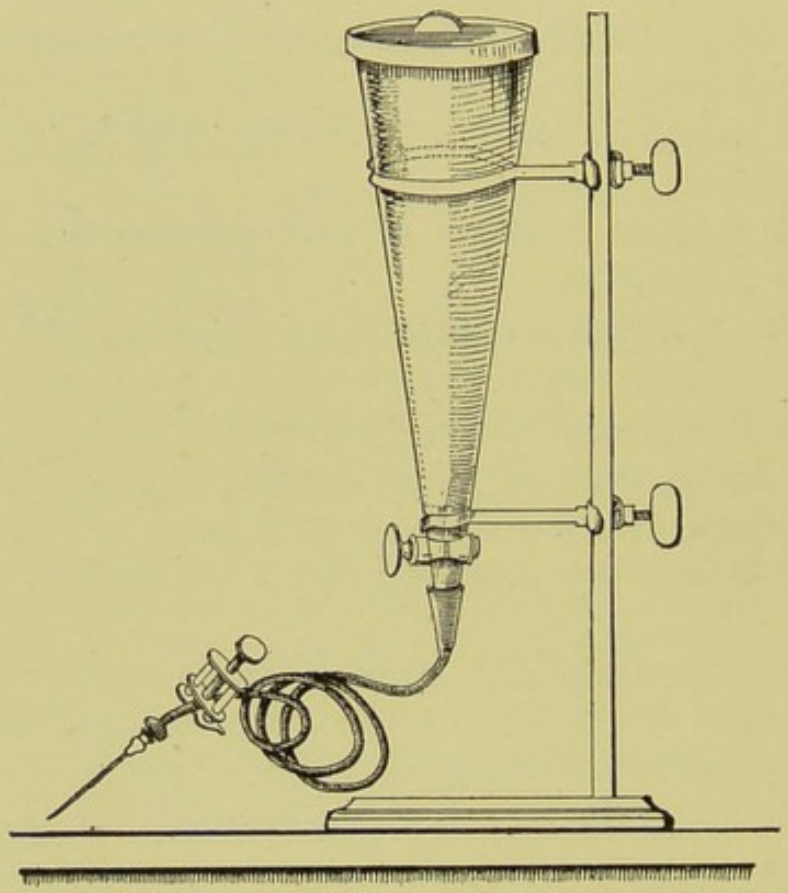


Fig. 141.—Conical separatory funnel, fitted for injection of fluid cultivations.

needle and provided with a screw clamp, is now attached to the nozzle of the funnel and the operation completed according to the requirements of the particular case.

If the injection has to be made into the subcutaneous tissue the "fall" may not be sufficient to force the fluid in. In this case it will be necessary to transfer the culture to a sterile wash-bottle and fasten a rubber

hand bellows to the air inlet tube (interposing an air filter) and attach the tubing with the injection needle to the outlet tube (Fig. 142). By careful use sufficient force can be obtained to drive the injection in.

(*b*) *Cultivations on Solid Media* (*e. g., Oblique Agar*).—

1. By means of a sterile graduated pipette introduce a suitable small quantity of sterile bouillon (or sterile normal saline solution) into the culture tube.

2. With a sterile platinum loop or spatula scrape the bacterial growth off the surface of the medium,

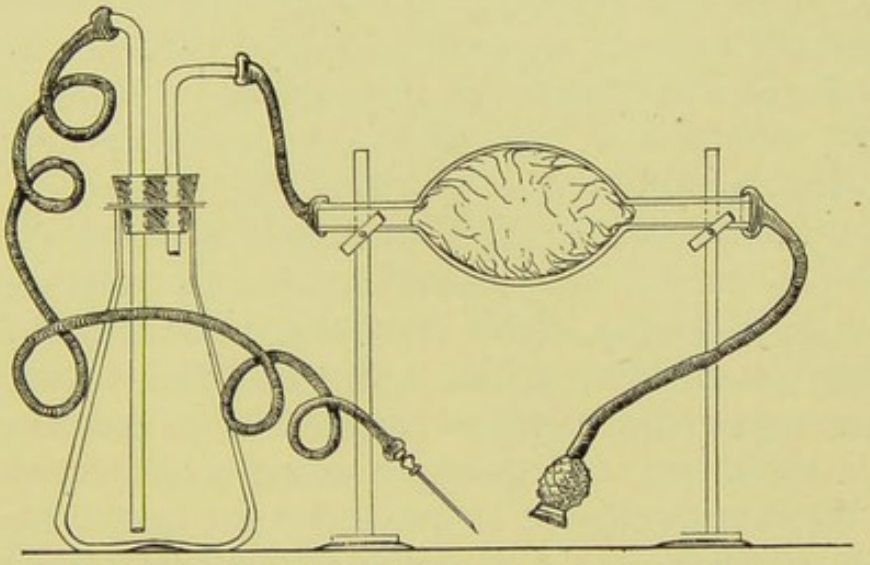


Fig. 142.—Arrangement of pressure injection apparatus.

and emulsify it with the bouillon. It then becomes to all intents and purposes a fluid inoculum.

3. Pour the emulsion into a sterile capsule and fill the syringe therefrom.

Minimal Lethal Dose: If the purpose of the inoculation is to determine the minimal lethal dose, a slightly different procedure is followed. For this purpose a special platinum loop must be employed, some 2.5 mm. by 0.75 mm., manufactured with parallel sides, and calibrated by careful weighing. (One can determine approximately by this method the amount of bacterial growth the loop will hold when filled.)

1. The cultivation must be prepared on a solid medium of the optimum reaction, incubated at the optimum temperature, and injected at the period of greatest activity and vigour, of the particular organism it is desired to test.

2. Arrange four sterile capsules in a row and label them I, II, III, and IV. Into the first deliver 10 c.c. sterile bouillon by means of a sterile graduated pipette; and into each of the remaining three, 9.9 c.c.

3. Remove one loopful of the bacterial growth from the surface of the medium in the culture tube, observing the usual precautions against contamination, and emulsify it evenly with the bouillon in the first capsule. Each cubic centimetre of the emulsion will now contain one-tenth of the organisms contained in the original loopful (written shortly 0.1 loop).

4. Remove 0.1 c.c. of the emulsion in the first capsule by means of a sterile graduated pipette and transfer it to the second capsule and mix thoroughly. Drop the infected pipette into a jar of lysol solution. This makes up the bulk of the fluid in the second capsule to 10 c.c., and therefore every cubic centimetre of bouillon in capsule II contains 0.001 loop.

5. Similarly, 0.1 c.c. of the mixture is transferred from capsule II to capsule III (1 c.c. of bouillon in capsule III contains 0.00001 loop), and then from capsule III to capsule IV (1 c.c. of bouillon in capsule IV contains 0.0000001 loop).

6. With sterile graduated pipettes remove the necessary quantity of bouillon corresponding to the various divisors of ten of the loop from the respective capsules, and transfer each "dose" to a separate sterile capsule and label; and to such doses as do not amount to 1 c.c. in bulk, add the necessary quantity of sterile bouillon.

7. Multiples of the loop are prepared by emulsifying 1, 2, 5, or 10 loops each with 1 c.c. sterile bouillon in separate sterile capsules.

8. Inoculate a series of animals with these measured doses, filling the syringe first from that capsule containing the smallest dose, then from the capsule containing the next smallest, and so on. If care is taken, it will not be found necessary to sterilise the syringe during the series of inoculations.

9. Plant tubes of gelatine or agar, liquefied by heat, from each of the higher dilutions, say from 0.0000001 loop to 0.01 loop; pour plates and incubate. When growth is visible enumerate the number of organisms present in each, average up and calculate the number of bacteria present in one loopful of the inoculum.

10. The smallest dose which causes the infection and death of the animal inoculated is noted as the minimal lethal dose (written shortly *m. l. d.*).

(*c*) *Toxins.*—Prepared by previously described methods (*vide* page 260), are manipulated in a similar manner to cultivations in fluid media.

(*d*) *Pathological Products.*—Fluid secretions, excretions, etc., such as serous exudation, pus, blood, etc., are collected direct from the body in sterile capillary pipettes (*vide* Fig. 10, page 21) in the following manner:

1. Open the case containing the pipettes, grasp one by the plugged end, remove it from the case, and replace the lid of the latter.

2. Pass the entire length of the pipette twice or thrice through the flame of the Bunsen burner.

3. Snap off the sealed end of the pipette with a pair of sterile forceps.

4. Thrust the point of the pipette into the secretion, apply the mouth to the plugged end, and fill the pipette by suction.

5. Seal the point of the pipette in the flame. (If using a pipette with a constriction below the plugged mouthpiece, this portion of the pipette may also be sealed in the flame.)

When about to perform the inoculation, snap the resealed end of the pipette off with sterile forceps and blow out the contents of the pipette into a sterile capsule, from which the injecting syringe is filled.

If the material when discharged into the capsule is very thick or viscous, a small quantity of sterile bouillon or normal saline solution may be used to dilute it, and thorough incorporation effected by the help of a sterile platinum rod.

Solid tissues, such as spleen, lymph glands, etc., may be divided into small pieces by sterile instruments and rubbed up in a sterilised mortar with a small quantity of sterile bouillon and the syringe filled from the resulting emulsion.

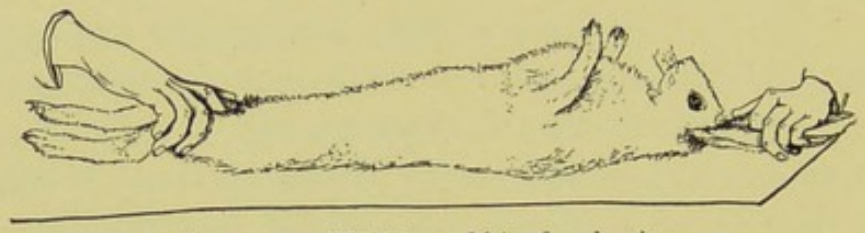


Fig. 143.—Holding rabbits for shaving.

If it is desired to inoculate tissue *en masse*, remove from the material a small cube of 1 or 2 mm. and introduce it into a wound made by sterile instruments in a suitable situation, and occlude the wounds by means of a sealed dressing.

Method of Securing Animals During Inoculation.—For the majority of inoculations, especially when no anæsthetic is administered, it is customary to employ an assistant to hold the animal (see Fig. 143); but when the animal is anæsthetised, it is more convenient to secure it firmly to some simple form of operating table, such as Tatin's (Fig. 144), which will accommodate rabbits, guinea-pigs, and rats.

A useful piece of apparatus, too, is Voge's holder for guinea-pigs, the method of using which is readily seen from the accompanying figure (Fig. 145).

The instrument itself consists of a hollow copper cylinder, one end of which is turned over a ring of stout copper wire, and from this open end a slot is cut

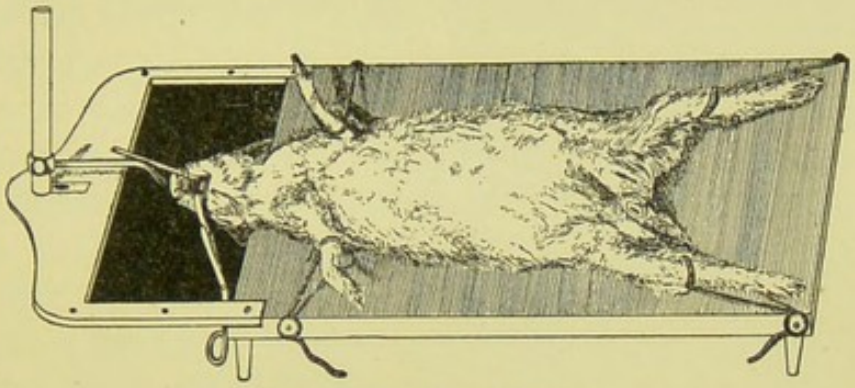


Fig. 144.—Operation table.

extending about half way along one side of the cylinder. The opposite end is closed by a "pull-off" cap and is perforated around its edge by a row of ventilating holes, which correspond with holes cut in the rim of the cap. In the event of the animal resisting attempts to remove it from the holder, backwards, this cap is taken off and the holder placed on the table and the guinea-pig allowed to walk out.

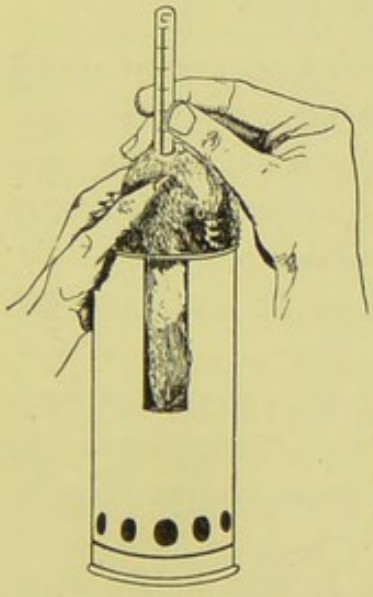


Fig. 145.—Taking guinea-pig's temperature.

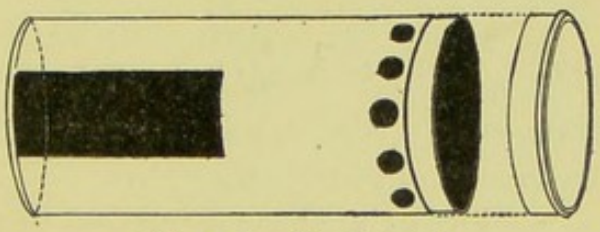


Fig. 146.—Voge's holder.

To provide for different-sized animals, two sizes of this holder will be found useful:

1. Length, 16 cm.; breadth, 6 cm.; size of slot, 8 cm. by 2.5 cm.

2. Length, 20 cm.; breadth, 8 cm.; size of slot, 10 cm. by 2.5 cm.

A convenient holder for mice and even small rats is shown in figure 147, the tail being securely held by

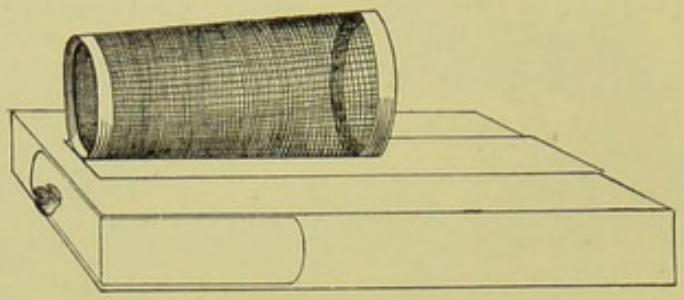


Fig. 147.—Mouse holder.

the spring clip. Needless to say, the holder should be entirely of metal, and the wire cage detachable and easily renewed.

METHODS OF INOCULATION.[1]

The following methods of inoculation apply more particularly to the rabbit, but from them it will readily be seen what modifications in technique, if any, are necessary in the case of the other experimental animals.

1. Cutaneous Inoculation.—(*Anæsthetic, none.*)

1. Have the animal firmly held by an assistant (or secured to the operating table).

2. Apply the liquid soap to the fur, over the area selected for inoculation, with a wad of cotton-wool, and lather freely by the aid of warm water; shave carefully and thoroughly.

3. Wash the shaved portion of skin thoroughly with 2 per cent. lysol solution.

4. Wash off the lysol with rectified spirit and allow the alcohol to evaporate.

[1] In the United Kingdom a special licence must be obtained from the Secretary of State for the Home Department, granting permission to its holder to perform inoculation upon the lower animals.

5. Make numerous short, parallel, superficial incisions with the point of a sterile scalpel.

6. When the oozing from the incisions has ceased, rub the inoculum into the scarifications by means of the flat of a scalpel blade, or a sterile platinum spatula.

7. Cover the inoculated area with a pad of sterile gauze secured *in situ* by strips of adhesive plaster or by sealing down the edges of the gauze with collodion.

8. Release the animal, place it in its cage, and affix a label upon which is written:

(a) Distinctive name or number of the animal.

(b) Its weight.

(c) Particulars as to source and dose of inoculum.

(d) Date of inoculation.

2. Subcutaneous Inoculation.—

(*a*) *Fluid Inoculum.—(Anæsthetic, none.)*

Steps 1–4. As for cutaneous inoculation.

5. Pinch up a fold of skin between the forefinger and thumb of the left hand; take the charged hypodermic syringe in the left hand, enter the needle into the ridge of skin between the finger and thumb, and push it steadily onwards until about 2 cm. of the needle are lying in the subcutaneous tissue. Now release the grasp of the left hand and slowly inject the fluid contained in the syringe.

6. Withdraw the needle, and at the same moment close the puncture with the left forefinger, to prevent the escape of any of the inoculum. The infected fluid, unless large in amount, will be absorbed within a very short time.

7. Label, etc.

(*b*) *Solid Inoculum.—(Anæsthetic, none.)*

Steps 1–4. As for cutaneous inoculation.

5. Raise a small fold of skin in a pair of forceps, and make a small incision through the skin with a pair of sharp-pointed scissors.

6. Insert a probe through the opening and push it

steadily onwards in the subcutaneous tissue, and by lateral movements separate the skin from the underlying muscles to form a funnel-shaped pocket with its apex towards the point of entrance.

7. By means of a pair of fine-pointed forceps introduce a small piece of the inoculum into this pocket and deposit it as far as possible from the point of entrance.

Or, improvise a syringe by sliding a piece of glass rod (to serve as a piston) into the lumen of a slightly shorter length of glass tubing and secure in position by a band of rubber tubing. Sterilise by boiling. Withdraw the rod a few millimetres and deposit the piece of tissue within the orifice of the tube, by means of sterile forceps. Now pass the tube into the depths of the "pocket," push on the glass rod till it projects beyond

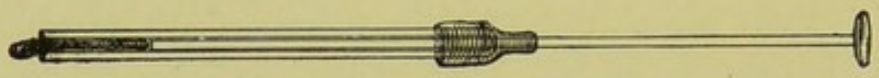


Fig. 148.—Glass tube syringe for subcutaneous "solid" inoculation.

the end of the tube, and withdraw the apparatus, leaving the tissue behind in the wound.

8. Close the wound in the skin with a dressing of gauze sealed with collodion (or tinct. benzoin), having previously inserted sutures, if necessary.

9. Label, etc.

3. Intramuscular.—

(*a*) *Fluid Inoculum.—(Anæsthetic, none.)*

Steps 1–4. As for cutaneous inoculation.

5. Steady the skin over the selected muscle or muscles with the slightly separated left forefinger and thumb.

6. Thrust the needle of the injecting syringe boldly into the muscular tissue and inject the inoculum slowly.

7. Label, etc.

(*b*) *Solid Inoculum.*—(*Anæsthetic, A. C. E.*)

1. Secure the animal to the operation table and anæsthetise.

2. Shave and disinfect the skin at the seat of operation.

3. Surround the field of operation by strips of gauze wrung out in 2 per cent. lysol solution.

4. Incise skin, aponeurosis, and muscle in turn.

5. Deposit the inoculum in the depths of the incision.

6. Close the wound in the muscle with buried sutures and the cutaneous wound with either continuous or interrupted sutures.

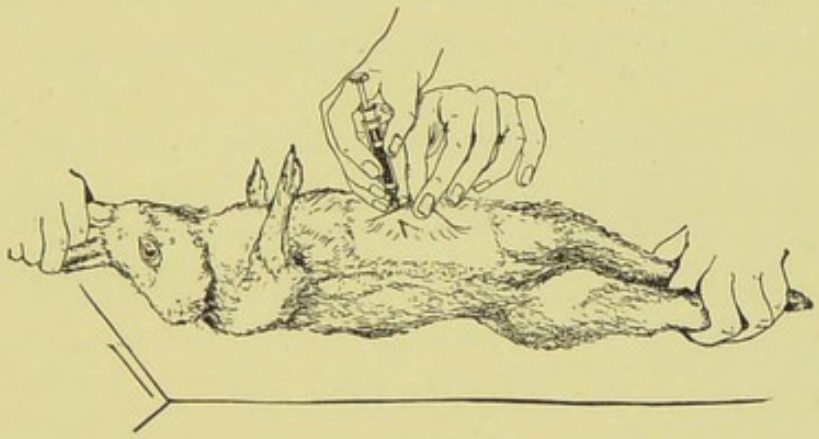


Fig. 149.—Intraperitoneal inoculation—fluid.

7. Apply a sealed dressing of gauze and collodion.

8. Remove the animal from the operating table.

9. Label, etc.

4. Intraperitoneal.—

(*a*) *Fluid Inoculum.*—(*Anæsthetic, none.*)

Steps 1–4. As for cutaneous inoculation. Shave a fairly broad transverse area, stretching from flank to flank.

5. Place the left forefinger on one flank and the thumb on the opposite, and pinch up the entire thickness of the abdominal parietes in a triangular fold. Now, by slipping the peritoneal surfaces (which are in

apposition) one over the other, ascertain that no coils of intestine are included in the fold.

6. Take the syringe in the right hand and with the needle transfix the fold near its base (Fig. 149).

7. Now release the fold, but hold the syringe steady; as the parietes flatten out, the point of the needle is left free in the peritoneal cavity.

8. Inject the fluid from the syringe.

9. Label, etc.

Second Method:

Steps 1–4. As in the first method.

5. Heat platinum searing wire (0.5 mm. wire, twisted to the shape indicated in figure 151, mounted in an aluminium handle) to redness, and with it burn

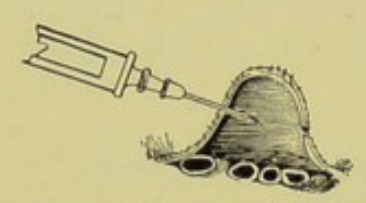


Fig. 150.—Section of abdominal wall, etc., showing point of needle lying free above the coils of intestine.

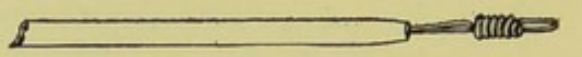


Fig. 151.—Platinum wire for burning hole through parietes.

a hole through the skin and abdominal muscle down to, but not through, the visceral peritoneum.

6. Fix a blunt-ended needle on to the charged syringe, and by pressing the rounded end firmly against the peritoneum it can easily be pushed through into the peritoneal cavity.

7. Inject the fluid from the syringe.

8. Label, etc.

This method is especially useful when it is desired to collect samples of the peritoneal fluid from time to time during the period of observation, as fluid can be removed from the peritoneal cavity, at intervals, through this aperture in the abdominal parietes, by means of a sterile capillary pipette.

(*b*) *Solid Inoculum* (or the inoculation of gelatine capsules [1] containing fluid cultivations).—(*Anæsthetic, A. C. E.*)

1. Anæsthetise the animal and secure it to the operating table.
2. Shave a large area of the abdominal parietes.
3. Make an incision through the skin in the middle line about 2 cm. in length, midway between the lower end of the sternum and the pubes.
4. Divide the aponeuroses between the recti upon a director.
5. Divide the peritoneum upon a director.
6. Introduce the inoculum into the peritoneal cavity.
7. Close the peritoneal cavity with Lembert's sutures.
8. Close the skin and aponeurosis incisions together with interrupted sutures, and apply a sealed dressing.
9. Release the animal from the operating table.
10. Label, etc.

[1] Collodion sacs may be readily prepared by the following method:

1. Dip a small test-tube (5 by 0.5 cm.), bottom downwards, into a beaker of collodion, and dry in the air; repeat this process three or four times.
2. Dip the tube, with its coating of collodion, alternately into a beaker of alcohol and one of water. This loosens the collodion and allows it to be peeled off in the shape of a small test-tube.
3. Take a 20 cm. length of glass tubing, of about the diameter of the test-tube used in forming the sac, and insert one end into the open mouth of the sac.
4. Suspend the glass tube with attached sac, inside a larger test-tube, by packing cotton-wool in the mouth of the test-tube around the glass tubing, and place in the incubator at 37° C. for twenty-four hours. When removed from the incubator, the sac will be firmly adherent to the extremity of the glass tubing.
5. Plug the open end of the glass tubing with cotton-wool, and sterilise the test-tube and its contents in the hot-air oven.

To use the sac, remove the plug from the glass tubing, partly fill the sac with cultivation to be inoculated, by means of a sterile capillary pipette, and replug the tubing. When the abdominal cavity has been opened, remove the tubing and attached sac from the protecting test-tube, close the sac by tying a sterilised silk thread tightly around it a little below the end of the glass tubing, and separate it from the tubing by cutting through the collodion above the ligature, and the sac is ready for insertion in the peritoneal cavity.

5. Intracranial.—(*Anæsthetic, A. C. E.*)

1. Anæsthetise the animal and secure it to the operating table, dorsum uppermost.

2. Shave a portion of the scalp immediately in front of the ears.

3. Mark out a crescentic flap of skin muscle, etc., convexity forwards, commencing 0.5 cm. in front of the root of one ear and terminating at a similar spot in front of the other ear. Reflect the marked flap.

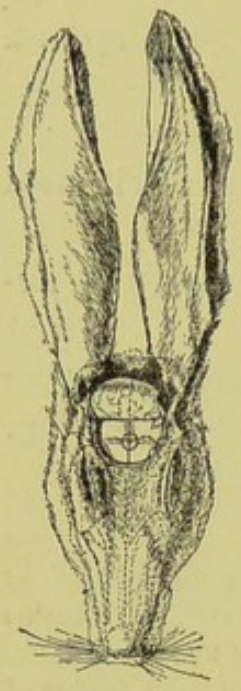


Fig. 152.—Intracranial rabbit.

4. With a small trephine (diameter 0.5 cm.) remove a circular piece of bone from the parietal segment. The centre of the trephine hole should be at the intersection of the median line and a line joining the posterior canthi (Fig. 152).

5. Introduce the inoculum by means of a hypodermic syringe, perforating the dura mater with the needle and depositing the material immediately below this membrane.

6. Turn back the flap of skin and secure it in position with interrupted sutures.

7. Dress with sterile gauze and wool and seal the dressing with collodion.

6. Intraocular.—

(*a*) *Fluid Inoculum.*—(*Anæsthetic, cocaine.*)

1. Instil a few drops of a sterile solution of cocaine, 2 per cent. (or B-eucaine, 2 per cent.), and repeat the instillation in two minutes.

2. Five minutes later have the animal firmly held by an assistant.

3. Steady the eye with fixation forceps; then pierce the cornea with the needle of the syringe and make the injection into the anterior chamber.

4. Label, etc.

(*b*) *Solid Inoculum.*—(*Anæsthetic, A. C. E.*)

1. Anæsthetise the animal and secure it firmly to the operating table.

2. Irrigate the conjunctival sac thoroughly with sterile saline solution.

3. Make an incision through the upper quadrant of the cornea into the anterior chamber by means of a triangular keratome.

4. Seize the solid inoculum in a pair of iris forceps, introduce it through the corneal wound, and deposit it on the anterior surface of the iris; withdraw the forceps.

5. Again irrigate the sac and the surface of the cornea.

6. Release the animal from the operating table.

7. Label, etc.

7. Intrapulmonary.—

Fluid Inoculum.—(*Anæsthetic, none.*)

1. Have the animal firmly held by an assistant. (In this case the foreleg of the selected side is drawn up by the assistant and held with the ear of that side.)

2. Shave carefully in the axillary line and disinfect the denuded skin.

3. Thrust the needle of the syringe boldly through the fifth or sixth intercostal space into the lung tissue.

4. Inject the contents of the syringe slowly.

5. Label, etc.

8. Intravenous.—

Fluid Inoculum.—(*Anæsthetic, none.*)

Vein selected, posterior auricular. Although this is smaller than the median vein, it is firmly bound down to the cartilage of the ear by dense connective tissue, and is therefore more readily accessible. (In the guinea-pig the jugular vein must be utilised, and in order to perform the inoculation satisfactorily a general anæsthetic must be administered to the animal.)

1. Have the animal firmly held by an assistant. The selected ear is grasped at its root and stretched forwards towards the operator.

2. Shave the posterior border of the dorsum of the ear.

3. Disinfect the skin over the vein, rubbing it vigourously with cotton-wool soaked in lysol. The friction will make the vein more conspicuous. Wash the lysol off with rectified spirit and allow the latter to evaporate.

4. Direct the assistant to compress the vein at the root of the ear. This will cause its peripheral portion to swell up and increase in calibre.

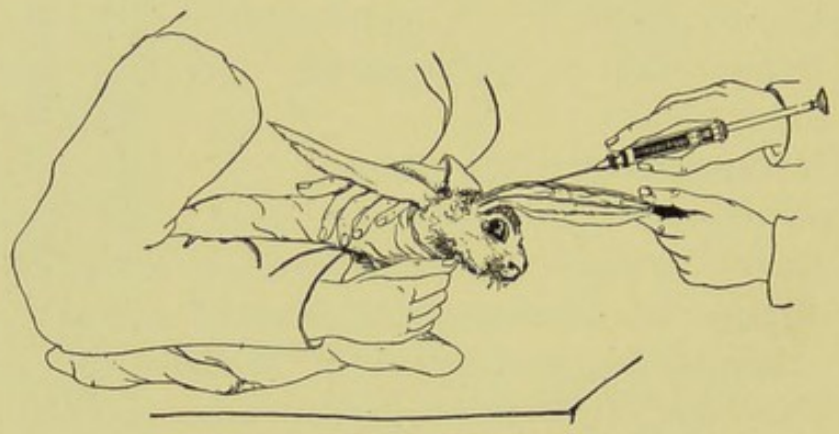


Fig. 153.—Intravenous inoculation.

Care must be taken in preparing the inoculum, as the injection of even small fragments may cause fatal embolism. To obviate this risk the fluid should, if possible, be filtered through sterile filter paper before filling into the syringe.

Air bubbles, when injected into a vein, frequently cause immediate death. To prevent this, the syringe after being filled should be held in the vertical position, needle uppermost. The needle should be thrust through the centre of a piece of sterile filter paper and the piston of the syringe pressed upwards until all the air is expelled from the barrel and needle. Should any

drops of the inoculum be forced out, they will fall on the filter paper, which should be immediately burned.

5. Hold the syringe as one would a pen and thrust the point of the needle through the skin and the wall of the vein till it enters the lumen of the vein (Fig. 153). Now press it onwards in the direction of the blood stream—*i. e.*, towards the body of the animal.

6. Direct the assistant to cease compressing the root of the ear, and *slowly* inject the inoculum. (If the fluid is being forced into the subcutaneous tissue, a condition which is at once indicated by the swelling that occurs, the injection must be stopped and another attempt made.)

7. Withdraw the needle.

8. Label, etc.

9. Inhalation.—

(*a*) *Fluid Inoculum.—(Anæsthetic, none.)*

1. Place the animal in a closed metal box.

2. Through a hole in one side introduce the nozzle of some simple spraying apparatus, such as is used for nasal medicaments.

3. Fill the reservoir of the instrument (previously sterilised) with the fluid inoculum, and having attached the bellows, spray the inoculum into the interior of the box.

4. On the completion of the spraying, open the box, spray the animal thoroughly with a 10 per cent. solution of formaldehyde (to destroy any of the virus that may be adhering to fur or feathers).

5. Transfer the animal to its cage.

6. Label, etc.

7. Thoroughly disinfect the inhalation chamber.

(*b*) *Fluid or Powdered Inoculum.—(Anæsthetic, A. C. E.)*

1. Anæsthetise the animal and secure it firmly to the operating table.

2. Pass a glass tube (previously sterilised) down the larynx into the trachea.

3. Connect the straight portion of a Y-shaped piece of tubing to the upper end of the sterilised tube and couple one branch of the Y to a separatory funnel containing the fluid inoculum, or insufflator containing the powdered inoculum, and the other to a hand bellows.

4. Allow the fluid inoculum to run into the lungs by gravity, or blow in the powdered inoculum by means of a rubber-ball bellows.

5. Remove the intratracheal tube; release the animal from the table.

6. Label, etc.

As an alternative method in the case of fairly large animals, such as rabbits, etc., a sterile piece of glass tubing of suitable diameter may be passed through the larynx down the trachea almost to its bifurcation. Fluid cultivations may then be literally poured into the lungs, or cultivations, dried and powdered, may be blown into the lung by the aid of a small hand bellows.

One other method of inoculation remains to be described, which does not require operative interference.

10. Feeding.—

1. *Fluid Inoculum.*—Small pieces of sterilised bread or sop (sterilised in the steamer at 100° C.) are soaked in the fluid inoculum and offered to the animals in a sterile Petri dish or capsule.

2. *Solid Inoculum.*—Small pieces of tissue are placed in sterile vessels and offered to the animals.

Raising the Virulence of an Organism. — If it is desired to raise or "exalt" the virulence of a feebly pathogenic organism, special methods of inoculation are necessary, carefully adjusted to the exigencies of

each individual case. Among the most important are the following:

1. *Passages of Virus.*—The inoculation of pure cultivations of the organism into highly susceptible animals, and passing it as rapidly as possible from animal to animal, always selecting that method of inoculation—*e. g.*, intraperitoneal—which places the organism under the most favorable conditions for its growth and multiplication.

2. *Virus Plus Virulent Organisms.*—The inoculation of pure cultivations of the organism together with pure cultivations of some other microbe which in itself is sufficiently virulent to ensure the death of the experimental animal, either into the same situation or into some other part of the body. By this association the organism of low virulence will frequently acquire a higher degree of virulence, which may be still further raised by means of "passages" (*vide supra*).

3. *Virus Plus Toxins.*—The inoculation of pure cultivations of the organism into some selected situation, together with the subcutaneous, intraperitoneal, or intravenous injection of a toxin,—*e. g.*, one of those elaborated by the proteus group,—either simultaneously with, before, or immediately after, the injection of the organism. By this means the natural resistance of the animal is lowered, and the organism inoculated is enabled to multiply and produce its pathogenic effect, its virulence being subsequently exalted by means of "passages."

Attenuating the Virulence of an Organism.—Attenuating or lowering the virulence of a pathogenic microbe is usually attained with much less difficulty than the exaltation of its virulence, and is generally effected by influencing the environment of the cultivations, as for example:

1. Cultivating in such media as are unsuitable by reason of their (*a*) composition or (*b*) reaction.

2. Cultivating in suitable media, but at an unsuitable temperature.

3. Cultivating in suitable media, but in an unsuitable atmosphere.

4. Cultivation in suitable media, but under unfavorable conditions as to light, motion, etc.

5. By a combination of two or more of the above methods.

XVI. POST-MORTEM EXAMINATIONS OF EXPERIMENTAL ANIMALS.

THE post-mortem examination should be carried out as soon as possible after the death of the animal, for it must be remembered that even in cold weather the tissues are rapidly invaded by numerous bacteria derived from the alimentary tract or the cavities of the body, and from external sources.

The following outlines refer to a complete and exhaustive necropsy, and in routine work the examination will rarely need to be carried out in its entirety. In all examinations, however, the searing irons must be freely employed, and it must be recollected that one instrument is only to be employed to seize or cut one structure. This done, it must be regarded as contaminated and a fresh instrument taken for the next step.

Apparatus Required:

Steriliser (*vide* page 38).
Surgical instruments: { Scissors. Forceps. Scalpels.
Spear-headed platinum spatula (Fig. 156).
Searing irons (Fig. 154).
Platinum loop.
Tubes of media—bouillon and oblique agar.
Grease pencil.
Sterile capillary pipettes.
Sterile capsules.
Cover-slips.
Bottles of fixing fluid (for pieces of tissue intended for sectioning).

1. Place the various instruments, forceps, scissors, scalpels, etc., needed for the autopsy inside the steriliser and sterilise by boiling for ten minutes; then raise

the tray from the steriliser, close the lid of the latter, and rest the tray on it.

2. Heat the searing irons to redness in a separate gas stove.

3. Fasten the body of the animal, ventral surface upwards (unless there is some special reason for having

Fig. 154.—Searing iron.

the dorsum exposed), out on a board by means of copper nails driven through the extremities.

4. Drench the fur (or feathers) with lysol solution, 2 per cent. This serves the twofold purpose of preventing the hairs from flying about and entering the body cavities during the autopsy, and of rendering innocuous any vermin that may be present on the animal.

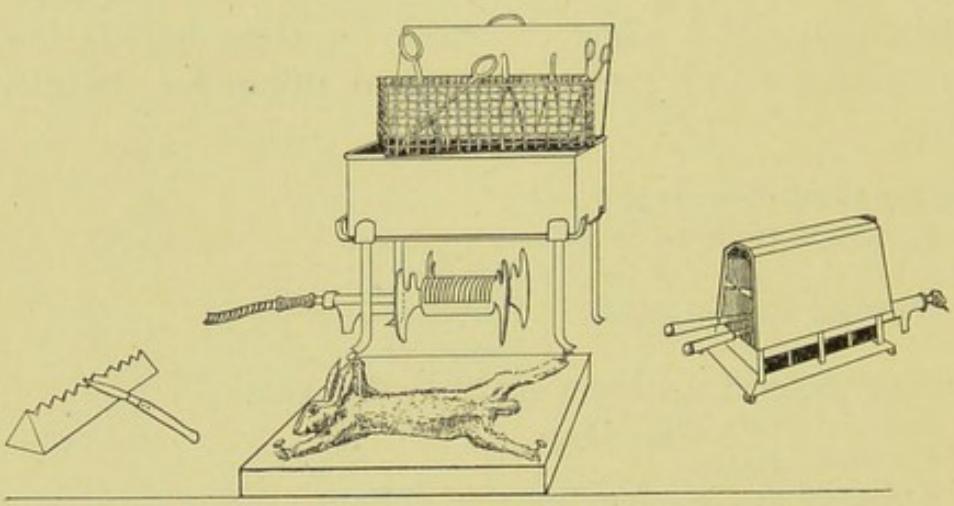


Fig. 155.—Apparatus for post-mortem examination, animal on board.

5. With sterile forceps and scalpel incise the skin in the middle line from the top of the sternum to the pubes. Make other incisions at right angles to the first out to the axillæ and groins, and reflect the skin in two lateral flaps. (Place the now infected instruments on the board by the side of the body or support them on a porcelain knife rest.)

6. Inspect the seat of inoculation. If any local lesion is visible, sear its exposed surface and remove material to make cultivations and cover-slip preparations from the deeper parts by means of the platinum loop. Collect specimens of pus or other exudation in capillary pipettes for subsequent examination.

7. Sear the whole of the exposed surface of the thorax with the searing irons.

8. Divide the ribs on either side of the sternum and remove a rectangular portion of the anterior chest wall with sterile scissors and a fresh pair of forceps, exposing the heart. Place the infected instruments by the side of the first set.

9. Raise the pericardial sac in a fresh pair of forceps and burn through this structure with a searing iron.

10. Grasp the apex of the heart in the forceps and sear the surface of the right ventricle.

11. Plunge the open point of a capillary pipette through the seared area into the ventricle and fill with blood.

Make cultivations and cover-slip preparations of the heart blood.

12. Sear a broad track in the middle line of the abdominal wall; open the peritoneal cavity by an incision in the centre of the seared line.

13. Collect a specimen of the peritoneal fluid (or pus, if present) in a capillary pipette. Make cultivations and cover-slip preparations from this situation.

14. Collect a specimen of the urine from the distended bladder in a large pipette (in the manner indicated for heart blood), for further examination, by cultivations, microscopical preparations, and chemical analysis.

15. Excise the spleen and place it in a sterile capsule. (Sear the surface of this organ; plunge the spear-headed spatula through the centre of the seared area, twist it round between the finger and thumb, and re-

move it from the organ. Sufficient material will be brought away in the eye in its head to make cultivations. A repetition of the process will afford material for cover-slip preparations.)

16. In like manner examine the other organs—liver, lungs, kidneys, lymphatic glands, etc. Prepare cultivations and cover-slip preparations.

17. Examine the other cavities of the body.

18. Remove small portions of various organs and place in separate bottles of "fixing fluid" for future sectioning. Affix to each bottle a label bearing all necessary details as to its contents.

19. If necessary, remove portions of the organs for preservation and display as museum specimens (*vide* page 292).

20. Gather up all the infected instruments, return

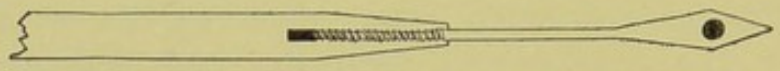


Fig. 156.—Spear-headed spatula.

them to the steriliser, and disinfect by boiling for ten minutes.

21. Cover the exposed cavities of the body with blotting or filter paper, moistened with 2 per cent. lysol solution.

22. Cremate the cadaver together with the board upon which it is fixed.

23. Stain the cover-slip preparations by suitable methods and examine microscopically.

24. Incubate the cultivations and examine carefully from day to day.

25. Make full notes of the condition of the various body cavities and of the viscera immediately the autopsy is completed; and add the result of the microscopical and cultural investigation when available.

26. Finally, the results of the action of the organism

or organisms isolated may be summarised under the following headings:

Tissue changes:[1]

1. Local—*i. e.*, produced in the neighbourhood of the bacteria.

Position: (*a*) At primary lesion.
(*b*) At secondary foci.

Character: (*a*) Vascular changes and tissue reactions. } Acute or chronic.
(*b*) Degeneration and necrosis. } Acute or chronic.

2. General (*i. e.*, produced at a distance from the bacteria, by absorption of toxins):
(*a*) In special tissues—*e. g.*, nerve cells and fibres, secreting cells, vessel walls, etc.
(*b*) General effects of malnutrition, etc.

Symptoms:
(*a*) Associated with known tissue changes.
(*b*) Without known tissue changes.

Permanent Preparations—Museum Specimens.—*I. Tube Cultivations of Bacteria.*—When showing typical appearances these may be preserved, if not permanently, at least for many years, as museum specimens, by the following method:

1. Take a large glass jar 25 cm. high by 18 cm. diameter, with a firm base and a broad flange, carefully ground, around the mouth. The jar must be fitted with a disc of plate glass ground on one side, to serve as a lid.

2. Smear a layer of resin ointment (B. P.) on the flange around the mouth of the jar.

3. Cover the bottom of the jar with a layer of cotton-wool and saturate it with formalin.

4. Remove the cotton-wool plug from the culture tubes and place them, mouth upwards, inside the jar. (If water of condensation is present in any of the culture tubes, it should be removed by means of a

[1] This table is adapted from Muir and Ritchie's "Bacteriology."

capillary pipette before placing the tubes in the formalin chamber.)

5. Adjust the glass disc, ground side downwards, over the mouth of the jar and secure it by pressing it firmly down into the ointment, with a rotary movement.

6. Remove the tubes from the formalin chamber after the lapse of a week, and dry the exterior of each.

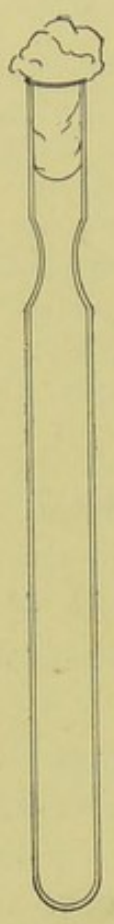


Fig. 157.—Bulloch's tubes.

7. Seal the open mouth of each tube in the blowpipe flame and label.

If the cultivations are intended for museum purposes when they are first planted, it is more convenient to employ Bulloch's tubes.

These are slightly longer than the ordinary tubes, and are provided with a constriction some 2 cm. below the mouth (Fig. 157)—a feature which renders sealing in the blowpipe flame an easy matter.

II. Tissues.—The naked-eye appearances of morbid tissues may be preserved by the following method:

1. Remove the tissue or organ from the cadaver, using great care to avoid distortion or injury.

2. Place it in a wide-mouthed stoppered jar, large enough to hold it conveniently, resting on a pad of cotton-wool, and arrange it in the position it is intended to occupy (but if it is intended to show a section of the tissue or organ, do not incise it yet).

3. Cover with the Kaiserling solution, and stopper the jar.

Kaiserling solution is prepared as follows:

Weigh out

Potassium acetate	30 grammes
Potassium nitrate	10 "

and dissolve in

Distilled water	750 c.c.

then add

Formalin	300 "

Filter.

4. After immersion in the formalin solution for twenty-four to forty-eight hours (according to size), transfer the tissue to a bath of methylated spirit for ten minutes.

5. Remove to a fresh bath of spirit and watch carefully. Immediately the natural colours show in their original tints, transfer to the mounting solution.

The mounting solution consists of

Glycerine	500 c.c.
Distilled water	500 "
Formalin	1 "

6. After twenty-four hours in this solution transfer to a museum jar, fill with fresh mounting solution, and seal.

6*a*. Or transfer to museum jar and fill with liquefied gelatine, to which has been added 1 per cent. formalin. Cover the jar and allow the gelatine to set. When solid, seal the jar in the usual manner.

XVII. OUTLINES FOR THE STUDY OF THE PATHOGENIC BACTERIA.

(The outlines here given for the study of the pathogenic bacteria are those in use in the author's elementary classes for medical and dental students, and for those qualifying for the Public Health Service. They represent the minimum of individual practical work necessary for the acquisition of a sound knowledge of the more important specific organisms of disease. At the same time, it cannot be too strongly urged that every student should, if possible, work out each organism completely and thoroughly on the lines suggested in the Scheme for Study, on page 205.)

The student who has conscientiously worked out the methods, etc., previously dealt with is in a position to make accurate observations and to write precise descriptions of the results of such observations. He is, therefore, now entrusted with pure cultivations of the various pathogenic bacteria, in order that he may study the life-history of each and record the results of his own observations—to be subsequently corrected or amplified by the demonstrator. In this way he is rendered independent of text-book descriptions, the statements in which he is otherwise too liable to take for granted, without personally attempting to verify their accuracy. For this reason none of the bacteria referred to in the following pages is described in detail, nor are any photomicrographic reproductions inserted.

During the course of this work attention is also directed, as occasion arises, to such other bacteria, pathogenic or saprophytic, as are allied to the par-

ticular organisms under observation, or so resemble them as to be possible sources of error, by working them through on parallel lines. By this means a fund of information is obtained with regard to the resemblances and differences, morphological and cultural, of a large number of bacteria.

The Organisms of Suppuration.—Whilst nearly all the pathogenic bacteria possess the power, under certain conditions, of initiating purely pyogenic processes in place of or in addition to their specific lesions (*e. g.*, the Bacillus tuberculosis, the pneumococcus, etc.), there are a certain few organisms which commonly express their pathogenicity in the formation of pus. These are usually grouped together under the title of "pyogenic bacteria," as distinct from those which only occasionally exercise a pyogenic rôle.

The organisms included under this heading are:

1. Staphylococcus pyogenes albus.
2. Staphylococcus pyogenes aureus.
3. Staphylococcus pyogenes citreus.
4. Streptococcus pyogenes longus.
5. Micrococcus tetragenus.
6. Micrococcus gonorrhœæ.
7. Bacillus pyocyaneus.

Staphylococcus albus. *pyogenes* *Micrococcus candicans.*
Micrococcus agilis.
Staphylococcus aureus. *pyogenes*
Staphylococcus citreus. *pyogenes*

1. Prepare subcultivations from each:

Bouillon,
Agar streak,
Blood-serum,
} and incubate at 37° C.

Agar streak,
Gelatine stab,
Potato,
Litmus milk,
} and incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day. Note M. agilis refuses to grow at 37° C.

2. Make hanging-drop preparations from the bouillon and agar cultivations after twenty-four hours' incubation. Examine microscopically and compare.

3. Prepare cover-slip films from the agar cultures, after twenty-four hours' incubation. Stain for flagella by the modified Pitfield's method.

4. Make microscopical preparations of each from all the various media after twenty-four and forty-eight hours' and three days' incubation. Stain carbolic methylene-blue, carbolic fuchsin, Gram's method. Examine microscopically and compare.

5. Stain section of cardiac muscle provided (staphylococcus in ulcerative endocarditis) by Gram's method, and counterstain with eosin.

6. Stain film preparation of pus from an abscess (containing Staphylococcus pyogenes aureus) with carbolic methylene-blue and also by Gram's method, counterstained with eosin.

7. Inoculate a white mouse subcutaneously with three loopfuls of a forty-eight-hour agar cultivation

of the Staphylococcus aureus, emulsified with 0.2 c.c. sterile broth.

Observe carefully during life, and if death occurs make a careful post-mortem examination (page 287).

Gonococcus. *Micrococcus tetragenus.*

1. Prepare subcultivations of each:

Bouillon, Agar, Litmus milk, } and incubate at 37° C.

Gelatine streak, Potato, } and incubate at 20° C.

Observe the culture tubes from day to day.

Note that the gonococcus refuses to grow upon these media either at 20° C. or at 37° C.

2. Prepare duplicate sets of subcultivations of the gonococcus:

Ascitic (or serum) bouillon, Serum agar, Human blood agar. { Incubate one set at 37° C. and the other at 20° C.

Examine the culture tubes from day to day.

Note that the gonococcus refuses to grow at 20° C.

3. Prepare cover-slip films of the Micrococcus tetragenus from all the media and of the gonococcus from those media upon which it grows, after twenty-four hours' and three days' incubation.

Stain carbolic methylene-blue, Gram's method.

4. Double stain the section of mouse's spleen (Micrococcus tetragenus) with picrocarmine and Gram's method.

5. Stain cover-slip film preparations of urethral pus (containing gonococci) with carbolic methylene-blue, capsule stain, Gram's method, counterstained eosin.

6. Prepare three human serum agar plates (*vide*

page 198) in series from the specimen of urethral pus and incubate at 37° C. for thirty-six hours.

Endeavour to isolate the M. gonorrhœæ by subcultivating suspicious colonies in serum agar streak culture.

7. Inoculate a white mouse subcutaneously with two loopfuls of a two-day agar cultivation of the Micrococcus tetragenus.

Observe until death takes place, then make a complete post-mortem examination.

Streptococcus pyogenes longus.
Streptococcus of bovine mastitis.
Diplococcus pneumoniæ.
Streptococcus brevis.

1. Prepare subcultivations from each:

Agar streak,
Blood agar streak,
Bouillon,
Litmus milk,
Potato,
} and incubate at 37° C.

Gelatine streak,
Potato,
} and incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day.

Note that the pneumococcus refuses to grow at 20° C.

2. Prepare cover-slip films from each culture tube after twenty-four and forty-eight hours' incubation.

Stain carbolic methylene-blue, MacConkey's capsule stain, Gram's method, and compare.

3. Examine agar cultivations at five and fourteen days by means of stained preparations, for involution forms, so-called arthrospores, etc.

4. Test the agar cultures for spores after fourteen days' incubation. Result negative.

5. Stain film preparations from the specimen of pus (containing streptococcus) carbolic methylene-blue, Gram's method, counterstained eosin.

6. Double stain the sections of erysipelatous skin, with picrocarmine and Gram's method.

7. Make cover-slip films from the rusty sputum (from a case of pneumonia) and stain capsule stain, and Gram counterstained eosin.

8. Stain the section of pneumonia lung by the Gram-Weigert method, and counterstain with eosin.

9. Inoculate a rabbit intraperitoneally with a loopful of the blood agar cultivation of the pneumococcus.

Observe until death occurs, recording the rectal temperature at frequent intervals; then make a complete post-mortem examination.

Observe the capsulated diplococci in the heart blood.

10. Inoculate a white mouse subcutaneously with a loopful of the blood agar cultivation of the Streptococcus longus.

Observe during life, and when death occurs, make a complete post-mortem examination.

Bacillus of Friedländer. *B. of rhinoscleroma.*
(*Pneumobacillus.*)

1. Prepare subcultivations of each:

Bouillon, Agar streak, Blood-serum streak, Potato, Litmus milk.	Incubate at 37° C.
Gelatine streak, Gelatine stab, Glucose gelatine shake.	Incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day.

2. Make hanging-drop preparations from the agar cultures after twenty-four hours' incubation.

Examine microscopically, and compare.

3. Make film preparations of each, from all the various media after twenty-four hours' and three days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin, Gram's method. Examine microscopically and compare.

Note the pleomorphism and involution forms.

4. Test the agar cultures for spores after seven days' incubation. Result negative.

5. Stain the sections of mouse's kidney (containing pneumobacilli) with carbolic methylene-blue.

6. Stain the sections of mouse's spleen (containing pneumobacilli) with capsule stain.

7. Inoculate a mouse subcutaneously with a couple of loopfuls of a forty-eight-hour-old potato cultivation, emulsified with 0.2 c.c. sterile bouillon.

Observe during life, and after death make a complete post-mortem examination.

Note the well-defined capsules around the bacilli in the heart blood.

B. pyocyaneus. — *B. fluorescens liquefaciens.* — *B. fluorescens non-liquefaciens.*

1. Prepare subcultivations of each:

Bouillon, Agar streak, Blood-serum streak, Litmus milk.	Incubate at 37° C.
Agar streak, Gelatine stab, Potato.	Incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day.

Note the difference in the "optimum" temperatures.

2. Make hanging-drop preparations from the agar and bouillon cultures after twenty-four hours' incubation.

Examine microscopically, and compare.

3. Prepare cultivations of each:

(a) Glucose formate bouillon, and incubate anaerobically at 37° C. and 20° C.

Compare the culture tubes after three days' incubation.

(b) Nitrate bouillon, and incubate at 37° C.

Compare the gas production from day to day.

(c) Glycerine agar, and incubate at 37° C. and 20° C.

Compare the pigment production at five, ten, and fourteen days.

4. Endeavour to obtain solutions of the pigment, formed upon agar and blood-sérum in water, chloroform, alcohol.

5. Prepare cover-glass films from the agar cultivations at eighteen to twenty-four hours.

Stain by the modifield Pitfield method.

6. Make film preparations of each from the various media after twenty-four hours' and three days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin, Gram's method, and compare.

7. Stain the sections of guinea-pig's spleen (B. pyocyaneus) with carbolic methylene-blue.

8. Make a complete post-mortem examination of the body of the guinea-pig (septicæmia resulting from pyocyaneus infection).

Vibrio choleræ. *Vibrio metschnikovi.*
Vibrio of Finkler and Prior.
Spirillum rubrum.

1. Prepare subcultivations of each:

Bouillon,
Blood-serum streak,
Agar streak,
Peptone water,
Nitrate broth (1 per cent.),
Litmus milk.
} Incubate at 37° C.

Gelatine stab,
Potato.
} Incubate at 20° C.

Compare the naked-eye appearances of the cultures, from day to day.

2. Make hanging-drop preparations from the bouillon and agar cultures after eighteen hours' incubation.

Examine microscopically, and compare.

3. Prepare cover-glass films from the agar cultivations at eighteen to twenty-four hours.

Stain by the modified Pitfield method (for flagella).

4. Prepare cover-slip films from all the cultivations after twenty-four and forty-eight hours' and five days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin (diluted with water), by Gram's method. Examine microscopically, and compare.

5. Test the agar cultivations for spores after fourteen days' incubation at 37° C. Result negative.

6. Test the peptone water cultivation for indol and nitrite after two, three, and five days' incubation.

7. Test the nitrate bouillon for nitrites after three days' incubation.

8. Test a twenty-four-hour-old bouillon cultivation against the serum of a guinea-pig that has been immunised against the Vibrio choleræ.

9. Make a careful post-mortem examination of the guinea-pig (killed by intraperitoneal injection of the V. choleræ).

Bacillus diphtheriæ. (*Klebs-Löffler bacillus.*) *Bacillus of Hoffmann.* *Bacillus of xerosis.*

1. Prepare subcultivations of each:

Agar streak,
Blood-serum streak,
Potato,
Bouillon,
Litmus milk.
} Incubate at 37° C.

Gelatine streak. Incubate at 20° C.

Compare the naked-eye appearance of the cultures from day to day.

2. Make hanging-drop preparations from the bouillon and agar cultivations after twenty-four hours' incubation.

Examine microscopically, and compare.

3. Make cover-slip films from the blood-serum cultures, after twelve to eighteen hours' incubation.

Stain Neisser's method.

4. Make film preparations of each from all the media after twenty-four and forty-eight hours' and five days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin, Gram's method, Neisser's method, and compare.

5. Test the agar cultivations for spores, after fourteen days' incubation. Result negative.

6. Stain the sections of diphtheritic membrane (*a*) with carbolic methylene-blue, and (*b*) by the Gram-Weigert method—to demonstrate the fibrin.

Note the diphtheria bacilli in the superficial layers of the membrane, often arranged in clusters and masses.

7. Inoculate a guinea-pig under the skin of the abdomen with 1 c.c. of a forty-eight-hour-old bouillon cultivation of the B. diphtheriæ.

Observe carefully until death occurs, then make post-mortem examination.

Prepare cultivations on blood-serum from (*a*) local lesion, (*b*) heart blood, and incubate at 37° C.

Note that a cultivation from *a* yields a growth of the B. diphtheriæ, while that from *b* does not.

Prepare cover-glass films from local lesion. Note the bacilli present.

B. typhi abdominalis. *B. coli communis.*
B. enteritidis (of Gaertner).
B. aquatilis sulcatus.

1. Prepare subcultivations of each:

Bouillon, Litmus milk, Peptone water, Agar streak.	Incubate at 37° C.
Bouillon, Potato, Gelatine streak.	Incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day.

Note that B. aquatilis sulcatus will not grow at 37° C.

2. Make hanging-drop preparations from the bouillon and agar cultures after eighteen hours' incubation.

Examine microscopically, and compare.

3. Make cover-slip preparations from the agar cultivations after eighteen hours' incubation.

Stain modified Pitfield's method.

4. Make cover-slip preparations of each from all the media after twenty-four hours' and five days' incubation.

Stain carbolic methylene-blue, Gram's method, modified Pitfield's method, and compare.

5. Test agar cultivations at fourteen days for spores. Result negative.

6. Test the peptone water cultivations at five days for indol.

7. Prepare glucose-formate-gelatine "shake" cultivations and incubate at 20° C.

Compare from day to day.

8. Pour gelatine plate cultivations of each and incubate at 20° C., for three or four days.

Compare the colonies, naked eye and under a 1-inch lens.

9. Stain the sections of typhoid ulcer with carbolic methylene-blue.

10. Stain sections of guinea-pig spleen (B. typhi abdominalis) with dilute carbolic fuchsin.

11. Prepare a bouillon cultivation of each and incubate at 20° C. for twenty-four hours.

Test each culture against blood-serum from a typhoid patient.

Bacillus anthracis. *Bacillus subtilis.*
Bacillus mycoides.

1. Prepare subcultivations of each:

Bouillon,
Agar streak,
Litmus milk,
Blood-serum streak. } Incubate at 37° C.

Gelatine stab,
Potato. } Incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day.

2. Make hanging-drop preparations from the bouillon and agar cultivations after twenty-four hours' incubation.

3. Make cover-slip preparations from the agar cultivations after twenty-four hours' incubation.

Stain modified Pitfield, examine microscopically, and compare.

4. Make cover-slip preparations from the various

media after twenty-four and forty-eight hours' and seven days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin, Gram's method, and compare.

5. Prepare cultivations of each, in nitrate broth, incubate three days at 37° C., and test for nitrites.

6. Prepare cultivations of each, in peptone water, incubate three days at 37° C., and test for indol.

7. Test the agar cultivations for spores, after seven days' incubation.

8. Prepare cover-glass films from these agar cultivations and stain for spores by method 2 (*vide* page 91).

9. Double stain the sections of malignant pustule (from man, containing anthrax bacilli) with picro-carmine and Gram's method.

10. Stain the sections of guinea-pig's lung (containing B. anthracis) with carbolic methylene-blue.

11. Inoculate a guinea-pig subcutaneously with two loopfuls of a forty-eight-hour-old agar cultivation of the B. anthracis, emulsified with 1 c.c. sterile bouillon.

Observe carefully during life.

When dead, make complete post-mortem examination.

Bacillus tuberculosis. — *Bacillus of avian tubercle.* *Bacillus phlei.* (*Timothy grass bacillus.*)

1. Prepare subcultivations of the tubercle bacillus and that of avian tubercle:

Blood-serum streak,
Agar streak,
Glycerine agar streak,
Glycerine bouillon,
Litmus milk,
Glycerinated potato.
} Incubate at 37° C.

Glycerine agar streak,
Gelatine streak.
} Incubate at 20° C.

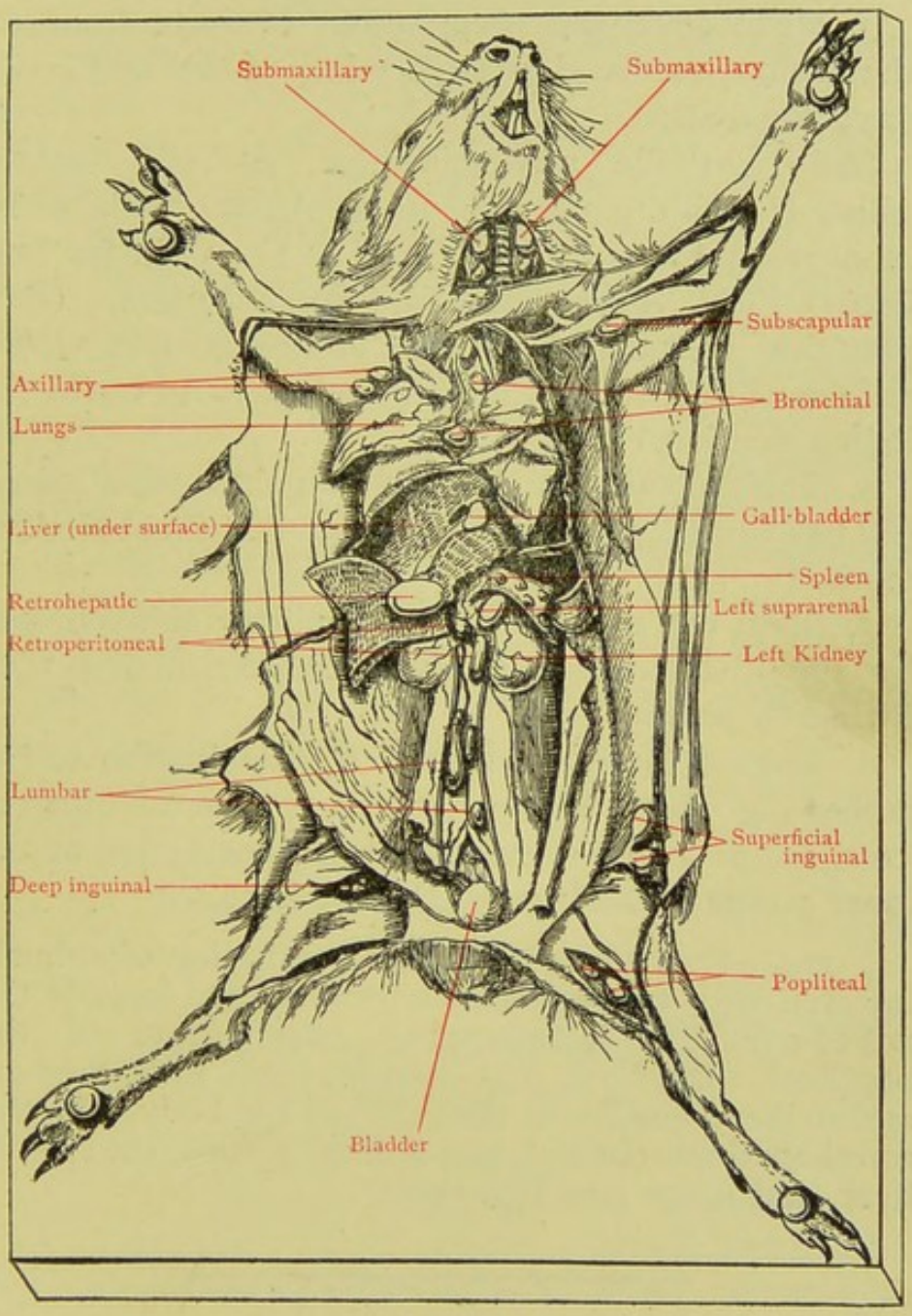

Submaxillary
Submaxillary
Subscapular
Axillary
Bronchial
Lungs
Gall-bladder
Liver (under surface)
Spleen
Retrohepatic
Left suprarenal
Retroperitoneal
Left Kidney
Lumbar
Superficial inguinal
Deep inguinal
Popliteal
Bladder

Fig. 158.

Compare the culture tubes from day to day.

Note that no growth takes place at 20° C.

2. Prepare subcultivations of the B. phlei upon all the ordinary media, and incubate at 37° C. and 20° C.

3. Make cover-slip preparations of each, from all the various media after twenty-four hours' and seven days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin (films of each organism should be stained in the usual manner, and also by immersion in the stain for four hours), Ziehl-Neelsen method, Gram's method. (Employ heat in staining with the aniline gentian violet, as is done in the Ziehl-Neelsen method, also warm the iodine solution.)

4. Double stain the sections of guinea-pig's spleen (B. tuberculosis) with picrocarmine and Ziehl-Neelsen method.

5. Stain the sections of lung (miliary tuberculosis) by the Ziehl-Neelsen method, counterstaining with Bismarck brown.

6. Make a careful post-mortem examination of the guinea-pig which has succumbed to general tuberculosis, as the result of subcutaneous inoculation at the inner aspect of the bend of the left knee.

NOTE.—Every post-mortem examination of animals infected with tuberculous material should include the naked-eye and microscopical examination of the popliteal, superficial, and deep inguinal, iliac, lumbar, and axillary glands on each side of the body, also the retrohepatic, bronchial, and sternal glands, the spleen, liver, and lungs (see Fig. 158).

Bacillus lepræ.

1. Stain the sections of (*a*) ulnar nerve and (*b*) leprous nodule by the Ziehl-Neelsen method

This organism cannot yet be cultivated artificially.

Bacillus tetani.
Bacillus œdematis maligni.
Bacillus of symptomatic anthrax.
B. enteritidis sporogenes.
B. botulinus.

1. Prepare agar streak subcultivations from each and incubate aerobically at 37° C.

Observe the culture tubes until the completion of seven days' incubation. Result, no growth.

2. Prepare bouillon cultivations of—

Bacillus tetani and immediately inoculate the culture tube with *B. prodigiosus.*

Bacillus œdematis maligni and immediately inoculate the culture tube with *Staphylococcus aureus.*

Bacillus of symptomatic anthrax and immediately inoculate the culture tube with *B. pyocyaneus.*

B. enteritidis sporogenes and immediately inoculate the culture tube with *B. subtilis.*

Incubate these cultivations aerobically at 37° C.

Observe the symbiotic growth of the anaerobes.

3. Prepare subcultivations from each:

Glucose formate agar streak, Blood-serum streak. } Incubate anaerobically at 37° C. (in Bulloch's apparatus).

Glucose formate bouillon, Glucose formate agar stab, Litmus milk. } Incubate anaerobically at 37° C. (in Buchner's tubes).

Glucose formate gelatine stab. } Incubate anaerobically at 37° C. (in Buchner's tube).

4. Make hanging-drop preparations in the ordinary way and examine microscopically.

Make another set of hanging-drop preparations, but before sealing the cell introduce a few crystals of pyrogallic acid and a drop of caustic soda solution, to absorb the oxygen and render the hanging drop "anaerobic." Examine microscopically.

5. Prepare cover-slip films from the agar streak cultivations at twenty-four hours.

Stain modified Pitfield (for flagella).

6. Prepare cover-slip films from each of the various media after forty-eight hours' and three days' incubation.

Stain carbolic methylene-blue and Gram's method.

7. Prepare cover-slip films from the agar cultivations after seven days' incubation.

Stain by Muller's method (for spores).

8. Make careful post-mortem examinations of the guinea-pigs which have died as the result of infection with the B. tetani, B. maligni, and B. enteritidis sporogenes, respectively.

Bacillus pestis.

B. septicæmiæ hæmorrhagicæ.

B. suipestifer.
(Bacillus of hog cholera.)

1. Prepare subcultivations from each:

Bouillon,
Agar streak, } and incubate at 37° C.
Litmus milk,

Gelatine streak,
Gelatine stab, } and incubate at 20° C.
Potato,

Compare the naked-eye characters of the cultures from day to day.

2. Add a loopful of sterile vaseline to a tube of nutrient bouillon.

Prepare a subcultivation of the B. pestis in this and incubate at 37° C.

Note the formation of stalactites depending from the fat globules.

3. Make hanging-drop preparations from the bouillon and agar cultures, after forty-eight hours' incubation. Examine microscopically and compare.

4. Prepare cover-slip film preparations of each from all the media after twenty-four, forty-eight hours' and three days' incubation.

Stain carbolic methylene-blue, carbolic thionine blue, Gram's method, and compare.

5. Prepare smear cultivations on the surface of very dry agar and incubate at 37° C. for forty-eight hours.

Make cover-slip preparations from the growth and stain with carbolic thionine blue.

Note the involution forms of B. pestis.

6. Examine the agar streak cultivations, after seven days' incubation, for spores.

7. Sterilise some salt, in a glass capsule, in the hot-air oven.

Add about 0.5 gramme to a tube of nutrient bouillon and incubate at 37° C. for forty-eight hours, to determine its sterility.

8. Inoculate this bouillon with the B. pestis and incubate at 37° C. for twenty-four hours.

Examine the resulting growth microscopically.

Note the plasmolysed bacilli.

9. Stain the sections of bubo (containing B. pestis) with (*a*) Löffler's methylene-blue and (*b*) carbolic thionine blue.

10. Make a complete post-mortem examination of the cadaver of the rat infected intraperitoneally with the B. pestis.

Streptothrix actinomycotica. *Cladothrix nivea.*

1. Prepare subcultivations from each:

Bouillon,
Glycerine bouillon,
Agar,
Glycerine agar,
Litmus milk,
} and incubate at 37° C.

Gelatine streak,
Potato,
} and incubate at 20° C.

Compare the naked-eye appearances of the cultures from day to day.

2. Prepare cover-slip film preparations of each from all the media after twenty-four hours' and three days' incubation.

Stain carbolic methylene-blue, carbolic fuchsin, Gram's method. Examine microscopically and compare.

3. Double stain sections of human liver (affected with actinomycosis) with picrocarmine and the Gram-Weigert method.

4. Stain the section of cow's tongue (infected with actinomyces) by Gram's method and counterstain with dilute aqueous solution of fuchsin.

5. Examine the pus from a breaking-down nodule (from cow's tongue) with a hand lens, pick out some of the minute yellowish granules with a sterile platinum loop, and transfer to a sterile capsule.

6. Crush the selected granules with a sterile glass rod. Make cover-slip preparations and stain by Gram's method.

7. Inoculate four tubes of glycerine agar in series with some of the material.

Observe the resulting growth macroscopically and microscopically.

B. influenzæ. *Bacillus ægyptiacus.*
(Koch-Week's bacillus.)

1. Prepare subcultivations of each:

Bouillon,
Serum bouillon,
Agar,
Blood agar,
Serum agar,
} and incubate at 37 C.

Observe from day to day.

Note that growth only occurs in serum and blood media.

2. Prepare subcultivations from each:

Serum bouillon,
Blood agar,
} and incubate at 20° C.

Observe from day to day for four days.

Note that no growth takes place.

3. Transfer the subcultivations (section 2) to the incubator at 37° C.

Note that no growth takes place—the bacilli are dead.

4. Prepare cover-slip films of each bacillus from all the media upon which growth takes place, after sixteen, twenty-four, and forty-eight hours' incubation.

Stain carbolic methylene-blue, very dilute aqueous solution of fuchsin, Gram's method. Examine microscopically and compare.

5. Prepare cover-slip preparations of the yellowish, more solid masses of the specimen of sputum from a case of influenza.

Stain in dilute fuchsin solution for twenty minutes. Examine microscopically.

6. Wash some of these selected portions of sputum in several changes of sterile distilled water.

7. Inoculate four blood agar tubes in series from the washed sputum, and incubate at 37° C.

Observe the resulting growth macroscopically and microscopically.

8. Prepare cover-slip films of pus from a case of acute muco-purulent conjunctivitis.

Stain carbolic methylene-blue, dilute fuchsin solution.

9. Inoculate four blood agar tubes in series from the pus from cases of acute conjunctivitis and incubate at 37° C. Observe the resulting growth macroscopically and microscopically.

Bacillus mallei.

1. Prepare subcultivations:

Bouillon,
Agar smear,
Blood-serum,
Litmus milk,
} and incubate at 37° C.

Gelatine streak,
Potato,
} and incubate at 20° C.

Observe the naked-eye appearances of the cultures from day to day.

2. Make hanging-drop preparations from the bouillon and agar cultivations.

3. Prepare cover-slip films from the agar and blood-serum cultivations, after eighteen hours' and three days' incubation.

Stain carbolic methylene-blue, Neisser's method, and by Gram's method. Examine microscopically and compare.

Also compare with corresponding preparations of the B. diphtheriæ.

4. Stain sections of the testicle of the guinea-pig (containing B. mallei), by overstaining with Nicollé's gentian violet (*vide* page 83), and subsequently decolourising with 1 per cent. acetic acid.

5. Stain the sections of horse's lung (containing glanders bacilli) with Löffler's methylene-blue.

6. Make a complete post-mortem examination of the guinea-pig killed by infecting subcutaneously with the B. mallei.

Micrococcus melitensis.

1. Prepare subcultivations:

Bouillon, Glycerine agar smear, Brain agar smear, Blood-serum, Litmus milk.	Incubate at 37° C.
Agar streak, Gelatine streak, Potato.	Incubate at 20° C.

Observe the naked-eye appearances of the cultures from day to day.

Note the extremely scanty growth on gelatine and agar at 20° C.

2. Make hanging-drop preparations from the bouillon and agar cultivations.

3. Prepare cover-slip films from the growth on each of the media and stain carbolic methylene-blue, dilute solution of fuchsin, by Gram's method; examine microscopically and compare.

Note the bacillary forms on agar.

4. Examine the agar cultivations, after seven days' incubation, for spores. Result negative.

5. Test a forty-eight-hour-old bouillon culture, filtered through a sterile filter paper, against the serum of a patient suffering from Malta fever.

XVIII. BACTERIOLOGICAL ANALYSES.

EACH bacteriological or bacterioscopical analysis of air, earth, sewage, various food-stuffs, etc., includes, as a general rule, two distinct investigations:

1. Quantitative.
2. Qualitative.

The first aims simply at enumerating (approximately) the total number of bacteria present in any given unit of volume irrespective of the nature and character of individual organisms.

The second seeks to classify the bacteria found, and to accurately identify individual organisms. As a subdivision of the qualitative examination, an estimation is often made, and with a fair degree of accuracy, of the numbers of some particular organism (*e. g.*, B. coli communis), present per unit, in the sample under examination.

The general principles underlying the bacteriological analyses of water, sewage, air and dust, soil, milk, ice cream, meat, and other tinned stuffs, as exemplified by the methods used by the author, are indicated in the following pages, together with the methods of testing filters and chemical germicides. It is hoped that the methods given will be found to be capable of expansion and adaptation to any circumstance or set of circumstances which may confront the student.

BACTERIOLOGICAL EXAMINATION OF WATER.

1. Quantitative.—

Collection of the Sample.—As the quantity of water actually used for this examination rarely exceeds 2 c.c., the most suitable vessels for the reception of the sample

are small glass bottles, 25 c.c. capacity, with narrow necks and overhanging glass stoppers (to prevent contamination of the bottle necks by falling dust). These must be carefully sterilised in the hot-air steriliser (*vide* page 35).

If the sample is obtained from a tap or pipe, turn on the water and allow it to run for a few minutes. Remove the stopper from the bottle and retain it in the hand whilst the water is allowed to run into the bottle and three parts fill it. Replace the stopper and tie it down, but *do not seal it.*

If the sample is obtained from a stream, tank, or reservoir, fasten a piece of stout wire around the neck of the bottle, remove the stopper, and retain it in the hand. Then, using the wire as a handle, plunge the bottle into the water, mouth downwards, until it is well beneath the surface; then reverse it, allow it to fill, and withdraw it from the water. Pour out a few cubic centimetres of water from the bottle, replace the stopper, and tie it down.

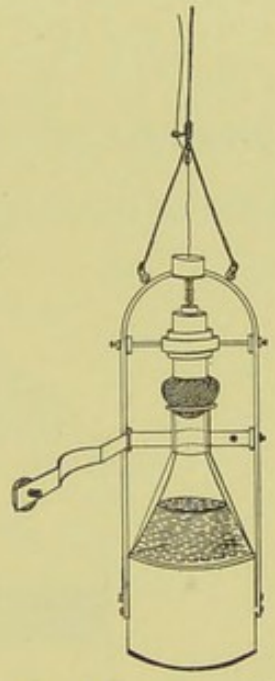


Fig. 159.—Esmarch's collecting bottle for water samples.

Or, use the apparatus designed by v. Esmarch (Fig. 159), in which the stopper can be removed, the bottle filled, and the stopper replaced, whilst the bottle is below the surface of the water, even at depths previously determined upon. When the apparatus is taken out of the water, the small bottles are filled from it, and packed in the ice-box mentioned below.

To prevent the multiplication of the bacteria contained in the water during transit from the place of collection to the laboratory, enclose the bottles, rolled in cotton-wool, in a double-walled metal box, pack the

space between the walls with pounded ice, close the metal box, and place it in a felt-lined wooden case (Fig. 160). (It has been shown that the majority of bacteria will survive exposure to the temperature of melting ice for some days, while practically none will multiply at this temperature.)

On reaching the laboratory, the method of examination consists in adding measured quantities of the

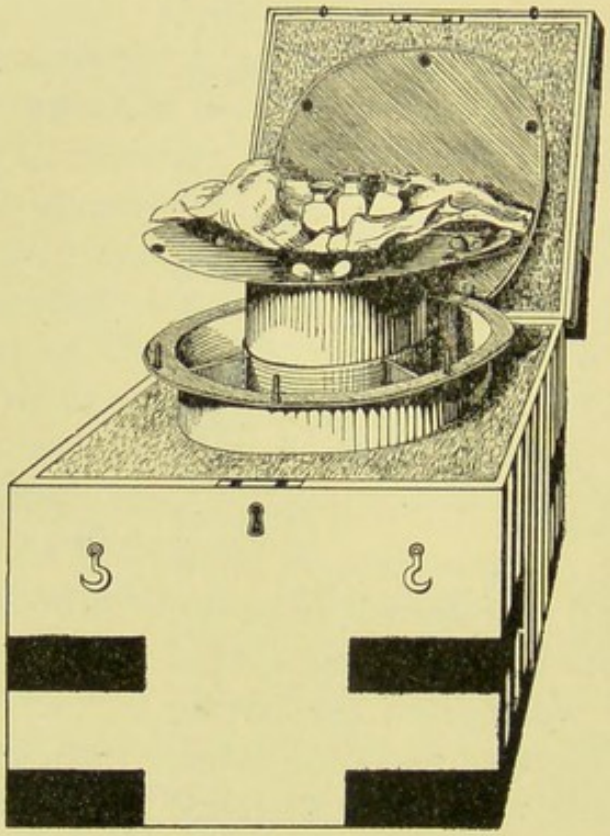


Fig. 160.—Ice-box.

water sample to several tubes of nutrient media previously liquefied by heat, pouring plate cultivations from each of these tubes, incubating at a suitable temperature, and finally counting the colonies which make their appearance on the plates.

The bacteria present in the water may comprise not only varieties which have their normal habitat in the water and will consequently be developed at 20° C., but also varieties which have been derived from,

or are pathogenic for, the animal body, and which will only develop well at a temperature of 37° C. In order to demonstrate the presence of each of these classes it will be necessary to plant both gelatine and agar plates.

Further, the sample of water may contain moulds, yeasts, or torulæ, and the development of these will be best secured by plating in wort gelatine and incubating at 20° C.

Apparatus Required:

Plate-levelling stand.
Case of sterile plates.
Case of sterile pipettes, 1 c.c. (in tenths of a cubic centimetre).
Case of sterile pipettes, 10 c.c. (in tenths of a cubic centimetre).
Case of sterile capsules, 25 c.c. capacity.
Tubes of nutrient gelatine.
Tubes of nutrient agar.
Tubes of wort gelatine.
One 250 c.c. flask of sterile distilled water.
Tall cylinder containing 2 per cent. lysol solution.
Bunsen burner.
Grease pencil.
Water-bath regulated at 42° C.

METHOD.—

1. Arrange the plate-levelling platform with its water compartment filled with water, at 45° C.

2. Number the agar tubes 1, 2, and 3; the gelatine tubes, consecutively, 1 to 6, and the wort tubes, 1, 2, and 3. Flame the plugs and see that they are not adherent to the lips of the tubes.

3. Place the agar tubes in boiling water until the medium is melted, then transfer them to the water-bath regulated at 42° C. Liquefy the nutrient gelatine and wort gelatine tubes by immersing them in the same water-bath.

4. Remove the bottle containing the water sample from the ice-box, distribute the bacterial contents

evenly throughout the water by shaking, cut the string securing the stopper, and loosen the stopper, but do not take it out.

5. Remove one of the 1 c.c. pipettes from the case, holding it by the plain portion of the tube. Pass the graduated portion twice through the Bunsen flame, raise the bottle containing the water sample from the bench in the other hand, grasp the stopper as if it were a cotton-wool plug, and remove it from the bottle with the hand holding the pipette; flame the mouth of the bottle.

6. Pass the pipette into the mouth of the bottle, holding its point well below the surface of the water. Suck up rather more than 1 c.c. into the pipette; empty the pipette by blowing. Now draw up exactly 1 c.c. into the pipette. Withdraw the pipette from the bottle, replace the stopper, and put the bottle down.

7. Take the first melted agar tube in the left hand, remove the cotton-wool plug, and add to its contents 0.5 c.c. of the water sample from the pipette; replug the tube and put it down. In a similar manner add 0.3 c.c. water to the contents of the second tube, and 0.2 c.c. to the contents of the third.

8. Drop the pipette into the cylinder containing lysol solution.

9. Mix the water sample with the medium in each tube in the manner described under plate cultivations; pour a plate from each tube. Label each plate with (*a*) the distinctive name or number of the sample, (*b*) the quantity of water sample it contains, and (*c*) the date.

10. Allow the plates to set, and incubate at 37° C.

11. Empty the water chamber of the levelling apparatus and refill it with ice-water.

12. By means of the sterile 10 c.c. pipette deliver 9.9 c.c. sterile distilled water into a sterile capsule.

13. Add 0.1 c.c. of the water sample to the 9.9 c.c.

sterile water in the capsule. This will give a dilution of 1 in 100.

14. Plant the six tubes of nutrient gelatine in the following manner: To the first tube add 0.5 c.c. of the water sample direct from the bottle; to the second, 0.3 c.c.; and to the third, 0.2 c.c.; and pour a plate of each tube. To the fourth tube add 0.5 c.c. of the diluted water sample from the capsule; to the fifth, 0.3 c.c.; and to the sixth, 0.2 c.c.; and pour a plate from each.

15. Label each plate with the quantity of the water sample it contains—that is, 0.5 c.c., 0.3 c.c., 0.2 c.c., 0.005 c.c., 0.003 c.c., and 0.002 c.c.

16. Allow the plates to set, and incubate at 20° C.

17. To the first tube of liquefied wort gelatine add 0.5 c.c. water sample; to the second, 0.3 c.c.; and to the third, 2 c.c.

18. Label the plates, allow them to set, and incubate at 20° C.

19. Count the number of colonies that have developed upon the agar at 37° C. after twenty-four hours' incubation.

20. Note the number of colonies present on each of the gelatine and wort gelatine plates after forty-eight hours' incubation.

21. Replace in the incubator, count again at three days, four days, and five days.

22. Calculate the number of organisms present per cubic centimetre of the original water from the average of the six gelatine plates.

Method of Counting.—The most accurate method of counting the colonies on each of the plates is by means of either Jeffer's or Pakes' counting disc. Each of these discs consists of a piece of paper, upon which is printed a dead black disc, subdivided by concentric circles and radii, printed in white. In Jeffer's counter, each subdivision has an area of 1 square centimetre;

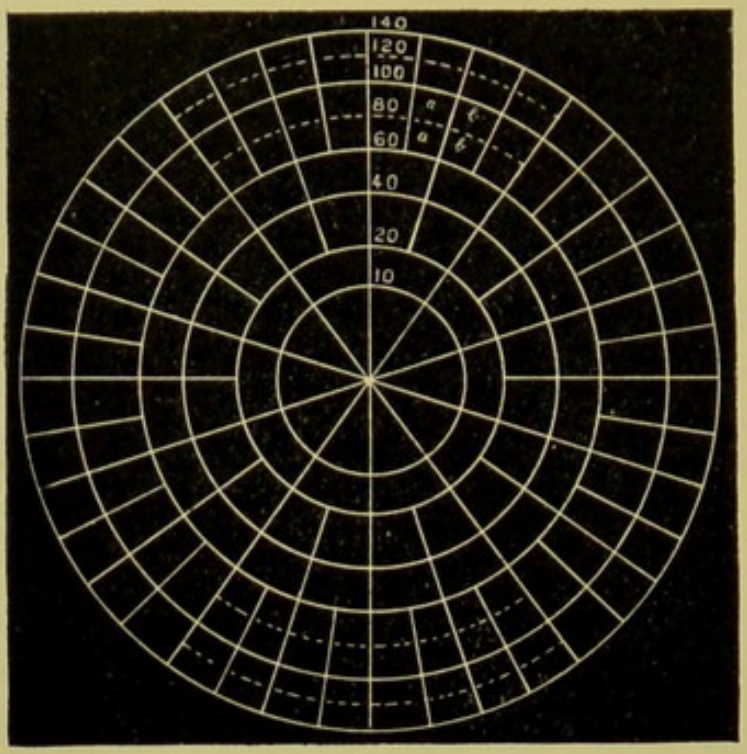


Fig. 161.—Jeffer's disc.

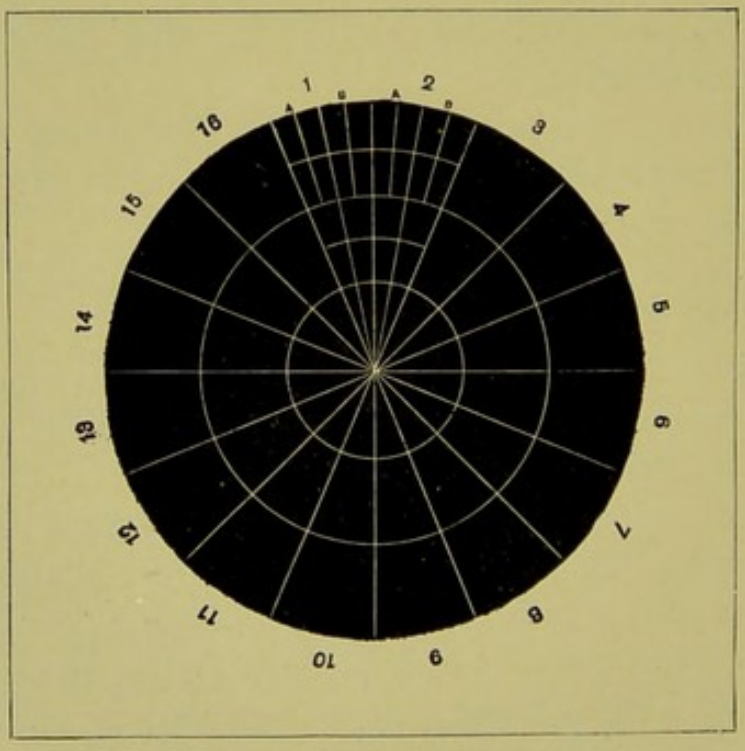


Fig. 162.—Pakes' disc.

in Pakes' counter, radii divide the circle into sixteen equal sectors, and counting is facilitated by equidistant concentric circles.

(a) In the final counting of each plate, place the plate over the counting disc, and centre it, if possible, making its periphery coincide with one or other of the concentric circles.

(b) Remove the cover of the plate, and by means of a hand lens count the colonies appearing in each of the sectors in turn. Make a note of the number present in each.

(c) If the colonies present are fewer than 500, the entire plate should be counted. If, however, they exceed this number, enumerate one-half, or one-quarter of the plate, or count a sector here and there, and from these figures estimate the number of colonies present on the entire plate.

It will be noted that the quantities of water selected for addition to each set of tubes of nutrient media total 1 c.c.; consequently the plates in a measure control each other; that is, the second and third plates of a series should together contain as many colonies as the first, and the second should contain about half as many more than the third.

2. Qualitative Examination.—

Collection of the Sample.—The quantity of water required for this examination is about 2000 c.c., and the vessel usually chosen for its reception is an ordinary blue glass Winchester quart bottle, sterilised in the hot-air oven, the stopper covered by a piece of sterile cotton-wool, and over this a paper or parchment cap fastened with string. The bottle may be packed in a wooden box or in an ordinary wicker case. The method of collecting the sample is identical with that described under the heading of Quantitative Examination; there is, however, not the same imperative

necessity to pack the sample in ice for transmission to the laboratory.

Examination.—The qualitative bacteriological examination of water is usually directed to the determination of the presence or absence of certain pathogenic organisms, usually one or more, but very rarely all, of those comprised in the following list:

I. B. coli communis.
II. B. typhi abdominalis.
III. B. enteritidis of Gaertner.
IV. B. enteritidis sporogenes.
V. Streptococci.
VI. Vibrio choleræ.
VII. B. anthracis.
VIII. B. tetani.

When these are present they are usually very highly diluted, and it is necessary, before commencing the examination, to adopt some means by which—

1. All the bacteria present in the sample of water, pathogenic or otherwise, may be concentrated in a small space.

2. The harmless non-pathogenic bacteria may be destroyed or their growth inhibited.

The first of these objects is usually effected by filtration of the water sample through a porcelain filter candle, and the subsequent emulsion of the bacterial residue of the original water with a small measured quantity of sterile bouillon.

The second is attained by so arranging the environment (*i. e.*, media, incubation temperature, and atmosphere) as to favor the growth of the pathogenic organisms at the expense of the harmless saprophytes.

Apparatus Required:

Sterile Berkfeld porcelain filter candle, fitted with rubber washer.

Rubber cork to fit the mouth of the filter candle, perforated with one hole.

Kitasato serum flask, 2000 c.c. capacity.

Fleuss air pump or water force pump.
Wulff's bottle, fitted as wash-bottle, and containing sulphuric acid (to act as a safety valve between filter and pump).
Pressure tubing, clamps, pinch-cock.
Retort stand, with ring and clamp.

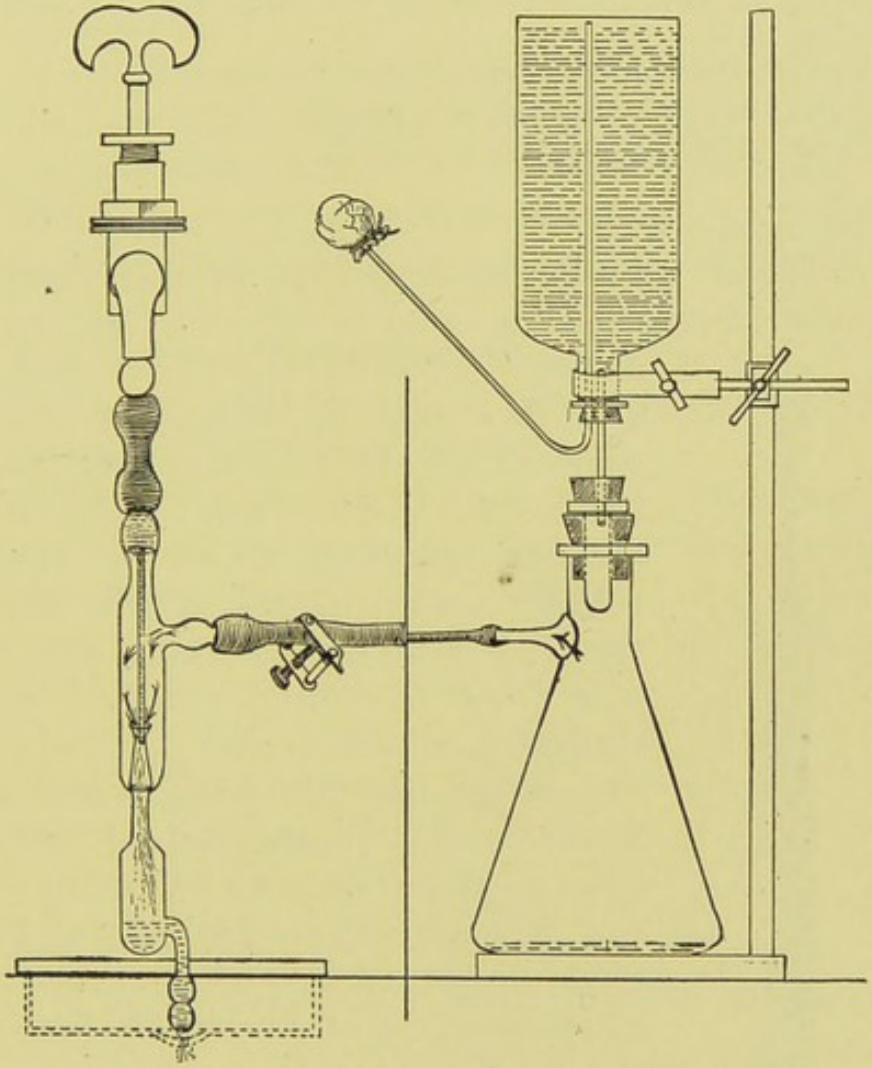


Fig. 163.—Water filtering apparatus. That portion of the figure to the left of the vertical line is drawn to a larger scale than that on the right, in order to show details of the force pump.

Rubber cork for the neck of Winchester quart, perforated with two holes and fitted with one 6 cm. length of straight glass tubing, and one V-shaped piece of glass tubing, one arm 32 cm. in length, the other 52 cm., the short arm being plugged with cotton-wool. The rubber stopper must be sterilised by boiling and the glass tubing by hot air, before use.

Flask containing 250 c.c. sterile broth.
Test-tube brush to fit the lumen of the candle, enclosed in a sterile test-tube (and previously sterilised by dry heat or by boiling).
Case of sterile pipettes, 10 c.c. in tenths.
Case of sterile pipettes, 1 c.c. in tenths.
Case of sterile pipettes, 1 c.c. in hundredths.
Tubes of various nutrient media (according to requirements).
Twelve Buchner's tubes with rubber stoppers.
Pyrogallic acid, 10 per cent. aqueous solution.
Dekanormal caustic soda solution.

METHOD.—

1. Fit up the filtering apparatus as in the accompanying diagram (Fig. 163), interposing the wash-bottle with sulphuric acid between the filter flask and the force-pump (in the position occupied in the diagram by the central vertical line), and placing another screw clamp on the rubber tubing connecting the lateral arm of the filter flask with the wash-bottle.

Fig. 164.—Sterile test-tube brush.

2. Filter the entire 2000 c.c. of water through the filter candle.

3. When the filtration is completed, screw up the clamps and so occlude the two pieces of pressure tubing.

4. Reverse the position of the glass tubes in the Wulff's bottle so that the one nearest the air pump now dips into the sulphuric acid.

5. Slowly open the metal clamps and allow air to gradually enter filter flask, having first passed through the acid, and restore the pressure.

6. Unship the apparatus, remove the cork from the mouth of the candle.

7. Pipette 10 c.c. of sterile broth into the interior of the candle, and by means of the sterile test-tube

brush (Fig. 164) emulsify the slimy residue which lines the candle, with the broth.

The entire bacterial contents of the original 2000 c.c. of water are now suspended in 10 c.c. of broth, so that 1 c.c. of the suspension is equivalent, so far as the contained organisms are concerned, to 200 c.c. of the original water.

Up to this point the method is identical, irrespective of the particular organism whose presence it is desired to demonstrate; but from this point onwards the methods must be specially adapted to the isolation of definite groups of organisms or of individual bacteria.

The Coli and Typhoid Groups.—

1. Number ten tubes of bile salt broth (*vide* page 169), consecutively from 1 to 10.

2. To each of the tubes of bile salt broth add varying quantities of the suspension by means of suitably graduated sterile pipettes, as follows:

No. 2	. . .	0.01	c.c.	(equivalent to	2	c.c.	of the	original	water	sample).
No. 3	. . .	0.02	"	"	4	"	"	"	"	"
No. 4	. . .	0.05	"	"	10	"	"	"	"	"
No. 5	. . .	0.1	"	"	20	"	"	"	"	"
No. 6	. . .	0.2	"	"	40	"	"	"	"	"
No. 7	. . .	0.3	"	"	60	"	"	"	"	"
No. 8	. . .	0.5	"	"	100	"	"	"	"	"
No. 9	. . .	1.0	"	"	200	"	"	"	"	"
No. 10	. . .	2.5	"	"	500	"	"	"	"	"

(To No. 1 should be added 1 c.c. of the original water sample before the filtration is commenced.[1])

3. Put up each tube anaerobically in a Buchner's tube and incubate at 42° C.

[1] If a positive result is obtained when using this method, it only needs a simple calculation to determine the smallest quantity (down to 1 c.c.) of the sample that contains at least one of the germs. For instance, if growth occurs in all the tubes from 4 to 10, and that growth is subsequently proved to be due to the presence of the B. coli, then it follows that at least one colon bacillus is present in every 10 c.c. of the water sample, but not in every 4 c.c. If, on the other hand, the presence of the B. coli can only be proved in tube No. 10, then the average number of colon bacilli present in the sample is two per litre.

4. Examine after twenty-four hours' incubation. Note in each culture tube:

(*a*) The presence or absence of visible growth.

(*b*) The reaction of the medium in each tube as indicated by the colour change (if any) the litmus has undergone.

(*c*) The presence or absence of gas formation as indicated by froth on the surface of the medium and the collection of gas in the fermentation tube.

5. Replace those tubes which show no signs of growth in the incubator. Examine after another period of twenty-four hours with reference to the same points.

6. Remove the culture tubes which show visible growth from the Buchner's tubes and make gelatine plate cultivations whether gas production and acid production are present or not. Incubate at 22° C. for forty-eight or seventy-two hours.

7. Pick off coliform or typhiform colonies and subcultivate in:

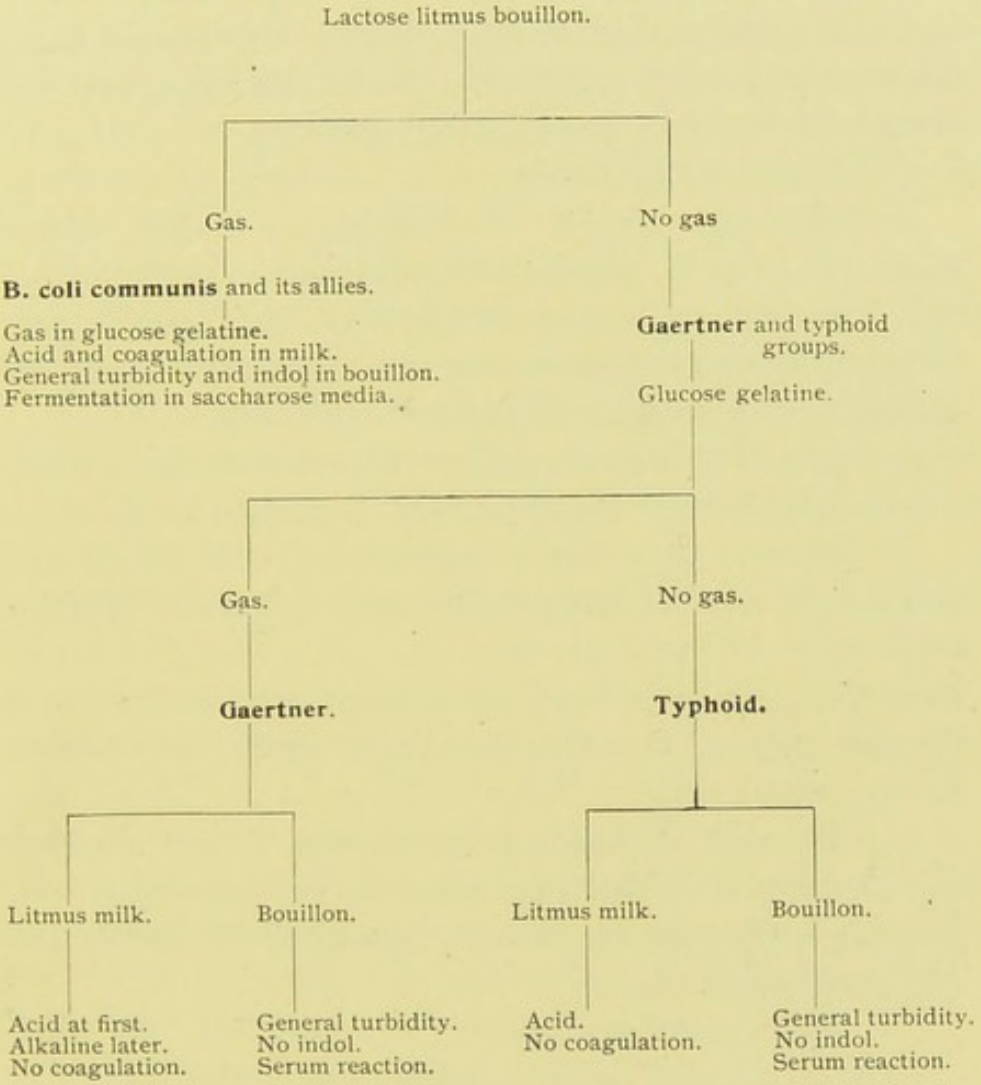
1. Nutrient bouillon.
2. Glucose formate bouillon.
3. Glycerine bouillon.
4. Lactose litmus bouillon.
5. Peptone water.
6. Litmus milk.
7. Glucose gelatine stab.
8. Lactose gelatine stab.
9. Maltose gelatine stab.
10. Neutral red-agar stab.
11. Nutrient gelatine streak.
12. Potato.

8. Differentiate by means of the characters of the resulting cultural reactions into members of the coli group, members of the Gaertner group, and members of the typhoid group.

9. Confirm these results by testing the organisms isolated with serum obtained from animals experi-

mentally protected against each of these groups of bacteria.

ANALYSIS OF MEMBERS OF THE COLI AND TYPHOID GROUPS.



Alternative Methods.—

(A) The Carbolic Method:

1. Take ten tubes of carbolised bouillon (*vide* page 144) and number them consecutively from 1 to 10.

2. Inoculate each tube with a different amount of the suspension, as in the previous method.

3. Incubate aerobically at 37° C.

4. Examine the culture tubes after twenty-four hours' incubation.

5. From those tubes which show signs of growth,

pour plates in the usual manner, using carbolised gelatine (*vide* page 149) in place of the ordinary gelatine, and incubate at 20° C. for three, four, or five days as may be necessary.

6. Subcultivate from any colonies that make their appearance, and determine their identity on the lines laid down in the previous method.

(B) Parietti's Method:

1. Take nine tubes of Parietti's bouillon (*vide* page 144)—*i. e.*, three each of those containing 0.1 c.c., 0.2 c.c., and 0.5 c.c. of Parietti's solution respectively. Mark plainly on the outside of each tube the quantity of Parietti's solution it contains.

2. To each tube add a different amount of the original water, or of the suspension, and incubate at 37° C.

3. Examine the culture tubes after twenty-four and forty-eight hours' incubation, and plate in gelatine from such as have grown.

4. Pick off suspicious colonies, if any such appear on the plates, subcultivate them upon the various media, and identify them.

(C) Elsner's Method: This method simply consists in substituting Elsner's potato gelatine (*vide* page 164) for ordinary nutrient gelatine in any of the previously mentioned methods.

(D) The Candle Method:

1. Remove the rubber stopper from the mouth of the filter candle, introduce 10 c.c. sterile bouillon into its interior, and emulsify the bacterial sediment; replug the mouth of the candle with a wad of sterile cotton-wool.

2. Remove the filter candle from the filter flask and insert it into the mouth of a flask or a glass cylinder containing sterile bouillon sufficient to reach nearly up to the rubber washer on the candle.

3. Incubate for twenty-four to thirty-six hours at 37° C.

4. From the now turbid bouillon in the glass cylinder pour gelatine plates and incubate at 20° C.

5. Subcultivate and identify any suspicious colonies that appear.

(The method depends upon the assumption that members of the typhoid and coli groups find their way through the porcelain filter from the interior to the surrounding bouillon at a quicker rate than the associated bacteria.)

B. Enteritidis Sporogenes.—

1. Transfer 5 c.c. of the emulsion from the filter candle to a sterile test-tube and plug carefully.

2. Place the test-tube in the interior of the benzole bath employed in separating out spore-bearing organisms (*vide* page 202), and expose to a temperature of 80° C. for twenty minutes.

3. Number ten tubes of litmus milk consecutively from 1 to 10.

4. Remove the test-tube from the benzole bath and shake well to distribute the spores evenly through the fluid.

5. To each tube of litmus milk add a measured quantity of the suspension corresponding to the amounts employed in isolating the coli group (*vide* page 327).

6. Put up each tube anaerobically in a Buchner's tube and incubate at 37° C.

7. Examine after twenty-four hours' incubation. Note (if the B. enteritidis sporogenes is present)—

(*a*) Acid reaction of the medium as indicated by the colour of the litmus or its complete decolourisation.

(*b*) Presence of clotting, and the separation of clear whey.

(*c*) Presence of gas, as indicated by fissures and bubbles in the coagulum, and possibly masses of coagulum driven up the tube almost to the plug.

8. Replace the tubes which show no signs of growth in the incubator for a further period of twenty-four

hours and again examine with reference to the same points.

9. Remove those tubes which give evidence of growth from the Buchner's tubes and carefully pipette off the whey; examine the whey microscopically.

10. Inoculate two guinea-pigs subcutaneously with 0.5 c.c. of the whey each and observe the result.

Streptococci.—

1. Melt ten tubes of nutrient agar in boiling water and cool to 42° C.

2. Number the tubes consecutively from 1 to 10.

3. To each of the tubes of liquefied agar add a measured quantity of the emulsion, corresponding to those amounts employed in isolating members of the coli group (*vide* page 327).

4 Pour plates in the usual manner and incubate aerobically at 37° C. for twenty-four hours.

5. Examine the plates carefully, pick off suspicious-looking colonies, and subcultivate in broth.

6. If the resulting growth appears, microscopically, to be composed of streptococci, subcultivate on the various media and identify.

Vibrio Choleræ.—

1. Number ten tubes of peptone water consecutively from 1 to 10.

2. To each of the tubes of peptone water add a measured quantity of the suspension, corresponding to those amounts employed in isolating the members of the coli group (*vide* page 327).

3. Incubate aerobically at 37° C. for twenty-four hours. Examine the tubes carefully for visible growth, especially delicate pellicle formation, which if present should be examined microscopically for vibrios.

4. Inoculate fresh tubes of peptone water from such of the tubes as exhibit pellicle formation,—from the pellicle itself,—and incubate at 37° C. for twenty-four hours.

5. Prepare gelatine and agar plates in the usual way from such of these tubes as show pellicle formation.

6. Pick off from the plates any colonies resembling those of the Vibrio choleræ and subcultivate upon all the ordinary laboratory media.

7. Test the vibrio isolated against the serum of an animal immunised to the Vibrio choleræ.

B. Anthracis.—

1. Transfer 5 c.c. of the emulsion from the filter candle to a sterile test-tube and plug carefully.

2. Place the test-tube in the interior of the benzole bath employed in separating out spore-bearing organisms (*vide* page 202), and expose to a temperature of 80° C. for twenty minutes.

3. Melt three tubes of nutrient agar in boiling water and cool to 42° C.

4. Number the tubes 1, 2, and 3. To No. 1 add 0.2 c.c., to No. 2 add 0.3 c.c., and to No. 3 add 0.5 c.c. of the suspension, and pour plates therefrom.

5. Incubate at 37° C. for twenty-four or forty-eight hours.

6. Pick off any colonies resembling those of anthrax and subcultivate on all the ordinary laboratory media.

7. Inoculate a *young* white rat subcutaneously (on the inner aspect of one of the hind legs) with two loopfuls of the cultivation on agar, emulsified with 1 c.c. sterile bouillon. Observe during life, and, if the animal succumbs, make a complete post-mortem examination.

B. Tetani.—

1. Proceed as detailed above in steps 1 and 2 for the isolation of the B. anthracis.

2. Add 1 c.c. of the suspension to each of three tubes of glucose formate broth, and incubate anaerobically in Buchner's tubes at 37° C.

3. From such of the tubes as show visible growth (with or without the production of gas) after twenty-

four hours' incubation inoculate guinea-pigs, subcutaneously (under the skin of the abdomen), using 0.1 c.c. of the bouillon cultivation as a dose. Observe carefully during life, and, if death occurs, make a complete post-mortem examination.

4. From the same tubes pour agar plates and incubate anaerobically in Bulloch's apparatus, at 37° C.

5. Subcultivate suspicious colonies on the various media, incubate anaerobically, making control cultivations on glucose formate agar, stab and streak, to incubate aerobically.

EXAMINATION OF SEWAGE AND SEWAGE EFFLUENTS.

Quantitative.—

Collection of the Sample.—As only small quantities of material are needed, the samples should be collected in a manner similar to that described under water for quantitative examination and transmitted in the ice apparatus used in packing those samples.

Apparatus Required.—As for water (*vide* page 319), substituting gelatine tubes for the agars and the wort gelatines.

METHOD.—

1. Arrange four sterile capsules in a row and number them I, II, III, IV.

2. Pipette 9 c.c. sterile bouillon into capsule No. I.

3. Pipette 9.9 c.c. sterile bouillon into capsules II, III, and IV.

4. Add 1 c.c. of the sewage to capsule No. I by means of a sterile pipette, and mix thoroughly.

5. Take a fresh sterile pipette and transfer 0.1 c.c. of the mixture from No. I to No. II and mix thoroughly.

6. In like manner transfer 0.1 c.c. from No. II to No. III, and then 0.1 c.c. from No. III to No. IV.

Now 1	c.c.	of	dilution	No.	I	contains	0.1	c.c.	of	the original	sewage.
1	"	"	"	"	II	"	0.001	"	"	"	"
1	"	"	"	"	III	"	0.00001	"	"	"	"
1	"	"	"	"	IV	"	0.0000001	"	"	"	"

7. Pour a set of gelatine plates from the contents of each capsule, three plates in a set, and containing respectively 0.2, 0.3, and 0.5 c.c. of the dilution. Label carefully; incubate at 20° C. for three, four, or five days.

8. Enumerate the organisms present in those sets of plates which have not liquefied, probably those from dilution III or IV, and calculate therefrom the number present per cubic centimetre of the original sample of sewage.

Qualitative.—The qualitative examination of sewage is but rarely required. When necessary, however, it is conducted on lines similar to those indicated under the corresponding section of water examination.

EXAMINATION OF AIR.

Quantitative.—

Apparatus Required:

Aspirator bottle, 10 litres capacity, fitted with a delivery tube, and having its mouth closed by a perforated rubber stopper, through which passes a short length of glass tubing.

Erlenmeyer flask, 250 c.c. capacity (having a wide mouth properly plugged with wool), containing 50 c.c. sterile bouillon.

Rubber stopper to fit the mouth of the flask, perforated with two holes, and fitted as follows:

Take a 15 cm. length of glass tubing and bend up 3 cm. at either end at right angles to the main length of tubing. Pass one of the bent ends through one of the perforations in the stopper; plug the opposite end with cotton-wool.

Take a glass funnel 5 or 6 cm. diameter with a stem 12 cm. in length and bend the stem close up to the apex of the funnel, in a gentle curve through a quarter of a circle; pass the long stem through the other perforation in the rubber stopper.

Rubber tubing.
Screw clamps and spring clips, for tubing.
Water steriliser.
Retort stand and clamps.
Apparatus for plating (as for enumeration of water organisms, *vide* page 319).

METHOD.—

1. Fill 10 litres of water into the aspirating bottle and attach a piece of rubber tubing with a screw clamp to the delivery tube. Regulate the screw clamp,

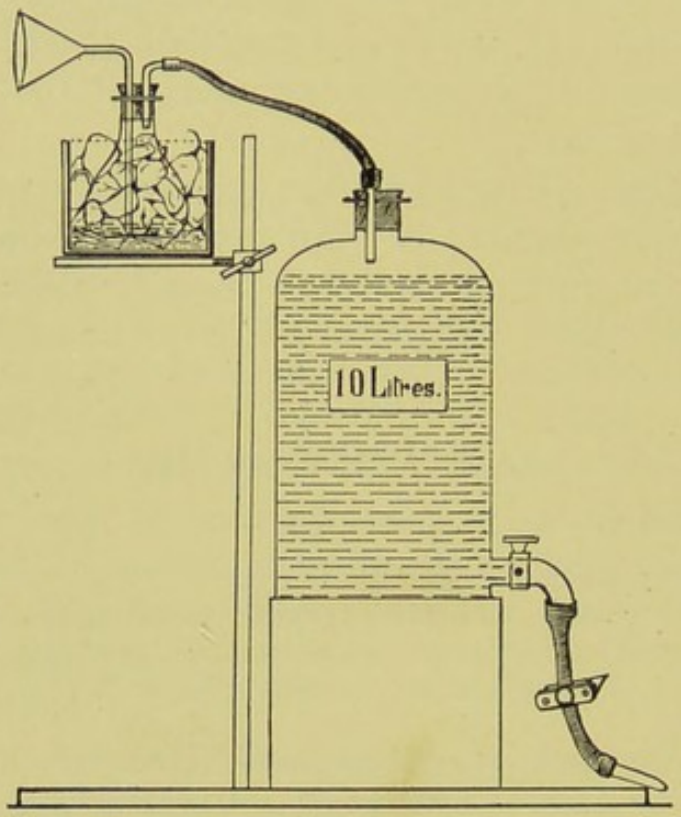


Fig. 165.—Arrangement of apparatus for air analysis.

by actual experiment, so that the tube delivers 1 c.c. of water every second.

At this rate the aspirator bottle will empty itself in just under three hours. Occlude the rubber tube below the screw clamp by means of a spring clip, and make up the contents of the aspirator bottle to 10 litres again.

2. Sterilise the fitted rubber cork, with its funnel

and tubing, by boiling in the water steriliser for ten minutes.

3. Remove the cotton-wool plug from the flask, and replace it by the rubber stopper with its fittings. Make sure that the end of the stem of the funnel is immersed in the bouillon.

4. Place the flask in a glass or metal vessel and pack it round with pounded ice. Arrange the flask with its ice casing just above the neck of the aspirator bottle.

5. Connect up the free end of the glass tube from the flask—after removing the cotton-wool plug—with the air-entry tube in the mouth of the aspirating bottle.

6. Remove the spring clip from the rubber tube, and allow the water to run.

Replenish the ice from time to time if necessary. (In emptying itself the aspirator bottle will aspirate 10 litres of air slowly through the broth in the Erlenmeyer flask.)

7. When the aspiration is completed, disconnect the flask and remove it from its ice packing.

8. Liquefy three tubes of nutrient gelatine and add to them 0.5 c.c., 0.3 c.c., and 0.2 c.c., respectively, of the broth from the flask, by means of a sterile graduated pipette, as in the quantitative examination of water. Pour plates.

9. Pour a second similar set of gelatine plates.

10. Incubate both sets of plates at 20° C.

11. Enumerate the colonies present in the two sets of gelatine plates after three, four, or five days and average the results from the numbers so obtained; estimate the number of micro-organisms present in 1 c.c., and then in the 50 c.c. of broth in the flask.

12. The result of air examination is usually expressed as the number of bacteria present per cubic metre (*i. e.*, kilolitre) of air; and as the number of organisms present in the 50 c.c. bouillon only represent those

contained in 10 litres of air, the resulting figure must be multiplied by 1000.

Qualitative.—

1. Proceed exactly as in the quantitative examination of air (*vide supra*), steps 1 to 10.

2. Pour plates of wort agar with similar quantities of the air-infected bouillon, and incubate at 37° C.

3. Pour plates of nutrient agar with similar quantities of the bouillon and incubate at 37° C.

4. Pour similar plates of wort gelatine and incubate at 20° C.

5. Pick off the individual colonies that appear in the several plates, subcultivate them on the various media, and identify them.

EXAMINATION OF SOIL.

Collection of Sample.—A small copper capsule 6 cm. high by 6 cm. diameter, with "pull-off" cap secured by a bayonet catch, previously sterilised in the hot-air oven, is the most convenient receptacle for samples of soil.

The instrument used for the actual removal of the soil from its natural position will vary according to

Fig. 166.—Soil scoop.

whether we require surface samples or soil from varying depths. In the first case, use an iron scoop, shaped like a shoe horn, but provided with a sharp spine (Fig. 166). This is wrapped in asbestos cloth and sterilised in the hot-air oven. When removed from the oven, wrap in a piece of oiled paper, silk, or gutta-

percha tissue, secured with string, as a further protection against contamination.

On reaching the spot whence the samples are to be taken, the coverings of the scoop are removed, and the asbestos cloth employed to brush away loose stones and débris from the selected area. The surface soil is then broken up with the point of the scoop, scraped up and collected in the body of the scoop, and transferred to the sterile capsule for transmission.

If it is desired to obtain samples of the earth from varying depths, some form of borer, such as that designed by Fraenkel (sterilised in a manner similar to that adopted for the scoop), must be employed for the purpose (Fig. 167).

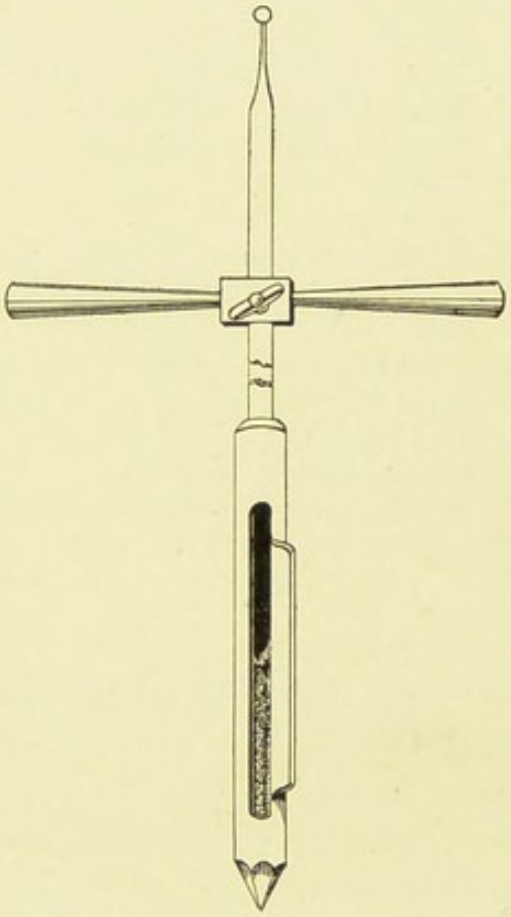


Fig. 167.—Fraenkel's borer.

Quantitative.—Four distinct investigations are included in the complete quantitative bacteriological examination of the soil:

1. The enumeration of the aerobic organisms.
2. The enumeration of the spores of aerobes.
3. The enumeration of the anaerobic organisms (including the facultative anaerobes).
4. The enumeration of the spores of anaerobes.

Further, by a combination of the results of the first and second, and of the third and fourth of these, the ratio of spores to vegetative forms is obtained.

Apparatus Required:

Case of sterile capsules (25 c.c. capacity).
Case of sterile graduated pipettes, 10 c.c. (in tenths of a cubic centimetre).
Case of sterile graduated pipettes, 1 c.c. (in tenths of a cubic centimetre).
Flask containing 250 c.c. sterile bouillon.
Tall cylinder containing 2 per cent. lysol solution.
Plate-levelling stand.
12 sterile plates.
Tubes of nutrient gelatine.
Tubes of wort gelatine.
Tubes of nutrient agar.
Tubes of glucose formate gelatine.
Tubes of glucose formate agar.
Water-bath regulated at 42° C.
Bunsen burner.
Grease pencil.
Sterile mortar and pestle (agate).
Sterile wide-mouthed Erlenmeyer flask (500 c.c. capacity).
Sterile metal funnel with short wide bore delivery tube to just fit mouth of flask.
Solid rubber stopper to fit the flask (sterilised by boiling).
Pair of scales.
Counterpoise (Fig. 88).
Sterile metal (nickel) spoon or spatula.
Fractional steriliser (Fig. 113).

METHOD.—

1. Arrange four sterile capsules numbered I, II, III, and IV; pipette 9 c.c. sterile bouillon into the first capsule, and 9.9 c.c. into each of the remaining three.

2. Pipette 100 c.c. sterile bouillon into the Erlenmeyer flask.

3. Remove the cotton-wool plug from the flask and replace it by the sterile funnel.

4. Place flask and funnel on one pan of the scales, and counterpoise accurately.

5. Empty the sample of soil into the mortar and triturate thoroughly.

6. By means of the sterile spatula add 10 grammes of the earth sample to the bouillon in the flask.

The final results will be more reliable if steps 2, 3, 4,

and 5 are performed under a hood—to protect from falling dust, etc.

7. Remove the funnel from the mouth of the flask; replace it by the rubber stopper and shake vigourously; then allow the solid particles to settle for about a minute.

8. Pipette off 1 c.c. of the supernatant bouillon, termed the "soil water," and add it to the contents of capsule I; mix thoroughly.

9. Remove 0.1 c.c. of the infected bouillon from capsule I and add it to capsule II, and mix.

10. In like manner add 0.1 c.c. of the contents of capsule II to capsule III and then 0.1 c.c. of the contents of capsule III to capsule IV.

Then 1	c.c.	fluid	from	capsule	I	contains	soil	water	from	.01	gm.	earth.
1	"	"	"	"	II	"	"	"	"	.0001	"	"
1	"	"	"	"	III	"	"	"	"	.000001	"	"
1	"	"	"	"	IV	"	"	"	"	.00000001	"	"

(*A*) *Aerobes* (*Vegetative Forms and Spores*).—

11. Pour a set of gelatine plates from the contents of each capsule—two plates in a set, and containing respectively 0.1 c.c. and 0.3 c.c. of the diluted soil water. Label and incubate.

12. Pour similar sets of wort gelatine plates from the contents of capsule II and III, label, and incubate at 20° C.

13. Pour similar sets of agar plates from the contents of capsules II and III; label and incubate at 37° C.

14. "Count" the plates after incubation for three, four, or five days, and from the figures thus obtained estimate—

(a) The number of aerobic micro-organisms present per gramme of the soil.

(b) The number of yeasts and moulds present per gramme of the soil.

(c) The number of aerobic organisms "growing at 37° C." present per gramme of the soil.

(*B*) *Anaerobes* (*Vegetative Forms and Spores*).—

15. Pour similar sets of plates in glucose formate gelatine and agar and incubate in Bulloch's anaerobic apparatus.

(*C*) *Aerobes and Anaerobes* (*Spores Only*).—

16. Pipette 5 c.c. soil water into a sterile tube.

17. Place in the differential steriliser at 80° C. for ten minutes.

18. Pour two sets of four gelatine plates containing 0.1, 0.2, 0.5, and 1 c.c. respectively of the soil water; label and incubate at 20° C., one set aerobically, the other anaerobically in Bulloch's apparatus.

19. "Count" the plates (delay the enumeration as long as possible) and estimate the number of spores of aerobes and anaerobes respectively present per gramme of the soil.

20. Calculate the ratio existing between spores alone and the total number of organisms.

Qualitative Examination.—The qualitative examination of soil is usually directed to the detection of one or more of the following:

I. Members of the coli or typhoid group.
II. Bacillus enteritidis sporogenes.
III. Streptococci.
IV. Bacillus anthracis.
V. Bacillus tetani.
VI. Bacillus œdematis maligni.
VII. The nitrous organisms.
VIII. The nitric organisms.

1. Transfer the remainder of the soil water (88 c.c.) to a sterile Erlenmeyer flask by means of a sterile syphon.

2. Fix up the filtering apparatus as for the qualitative examination of water, and filter the soil water.

3. Suspend the bacterial residue in 5 c.c. sterile bouillon (technique similar to that described for the water sample).

Every cubic centimetre of suspension now contains the soil water from nearly 1 gramme of earth.

The methods up to this point are identical no matter which organism or group of organisms it is desired to isolate; but from this stage onwards the process is varied slightly for each particular bacterium.

The Coli Group.—

Bacillus Enteritidis Sporogenes.—

Bacillus Anthracis.—

Bacillus Tetani.—

The methods adopted for the isolation of these organisms are identical with those already described under water (page 327 *et seq.*).

Bacillus Œdematis Maligni.—Method precisely similar to that employed for the B. tetani.

The Nitrous Organisms.—

The Nitric Organisms.—

1. Take six tubes of Warrington's solution (*vide* page 172) and number them consecutively from 1 to 6.

2. To tube No. 1 add 0.1 c.c. of the suspension.
" " " 2 " 0.2 " " " "
" " " 3 " 0.3 " " " "
" " " 4 " 0.5 " " " "
" " " 5 " 1.0 " " " "
" " " 6 " 2.5 " " " "

Label and incubate at 30° C.

3. Examine after twenty-four and forty-eight hours' incubation. From those tubes that show signs of growth make subcultivations in fresh tubes of the same medium and incubate at 30° C.

4. Make further subcultivations from such of those tubes as show growth, and again incubate.

5. If growth occurs in these subcultures, make surface smears on plates of Winogradsky's silicate jelly (*vide* page 172).

6. Pick off such colonies as make their appearance and subcultivate in each of these two media.

EXAMINATION OF MILK.

Quantitative.—

Collection of Sample.—"One-cow" milk, if taken from the apparently healthy animal (that is, an animal without any obvious lesion of the udder or teats) with ordinary precautions as to cleanliness, avoidance of dust, etc., contains but few organisms, and may be received directly into small sterile bottles (similar to those referred to under the collection of water for quantitative examination), packed in the ice-box for transmission, and dealt with in precisely the same manner as an ordinary water sample. In dealing with one-cow milk, from a suspected, or an obviously diseased animal, a complete analysis should include the examination (both qualitative and quantitative) of samples of (*a*) fore-milk, (*b*) mid-milk, (*c*) strippings, and, if possible, from each quarter of the udder, and the specimen should then be collected as described for mixed milk.

"Mixed" milk, on the other hand, by the time it leaves the retailer's hands, usually contains as many micro-organisms as an equal volume of sewage, and it becomes necessary to adopt special methods of collection, and, when collected, to estimate the number of its contained bacteria by the methods employed in the examination of sewage.

The apparatus used for the collection of a retail mixed milk sample consists of a cylindrical copper case, 16 cm. high and 9 cm. in diameter, provided with a "pull-off" lid, containing a milk dipper, also made of copper; and inside this, again, a wide-mouthed, stoppered glass bottle of 200 c.c. capacity (about 14 cm. high by 7 cm. diameter), having a tablet for notes, sand-blasted on the side. The copper cylinder and its contents, secured from shaking by packing with cotton-wool, are sterilised in the hot-air oven.

When collecting a sample,

1. Remove the cap from the cylinder.
2. Draw out the cotton-wool.
3. Lift out the bottle and dipper together.
4. Receive the milk in the sterile dipper, and pour it directly into the sterile bottle.
5. Enter the particulars necessary for the identification of the specimen, on the tablet, with a lead pencil, or pen and ink.
6. Repack the apparatus.

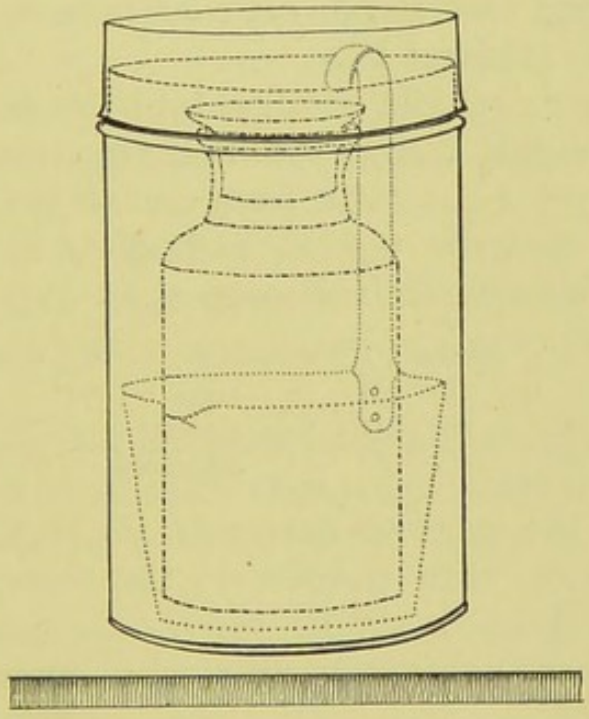


Fig. 168.—Milk-collecting bottle and dipper.

Four such bottles should be filled, so as to give a total of about 800 c.c. milk for examination. The four cases may be packed in an ice-box similar to, but larger than, that used for water specimens.

Apparatus Required:

Case of sterile capsules (25 c.c. capacity).
Case of sterile graduated pipettes, 10 c.c. (in tenths of a cubic centimetre).

Case of sterile graduated pipettes, 1 c.c. (in tenths of a cubic centimetre).
Flask containing 250 c.c. sterile bouillon.
Tall cylinder containing 2 per cent. lysol solution.
Plate-levelling stand.
Case of sterile plates.
Tubes nutrient gelatine agar (+ 10 reaction).
Tubes of wort gelatine.
Tubes of nutrient agar (+ 10 reaction).
Water-bath regulated at 42° C.
Bunsen burner.
Grease pencil.

METHOD.—

1. Arrange four sterile capsules in a row; number them I, II, III, and IV.

2. Fill 9 c.c. sterile bouillon into the first, and 9.9 c.c. bouillon into each of the three remaining capsules.

3. Remove 1 c.c. milk from one of the bottles by means of a sterile pipette and dilute it exactly as described for sewage (*vide* page 334).

Then 1 c.c. of	dilution	I	contains	0.1	c.c.	milk	sample.
1 " "	"	II	"	0.001	"	"	"
1 " "	"	III	"	0.00001	"	"	"
1 " "	"	IV	"	0.0000001	"	"	"

4. Melt the gelatine agar and the agar tubes in boiling water; then transfer to the water-bath and cool them down to 42° C.

5. Number the gelatine agar tubes consecutively 1 to 8.

6. To the first three tubes add 0.2 c.c., 0.3 c.c., and 0.5 c.c. respectively of the diluted milk from capsule IV.

7. To the second set of three tubes add similar quantities of the diluted milk from capsule III.

8. To the two remaining tubes add 0.1 c.c. and 0.2 c.c. of the diluted milk from capsule II.

9. Pour plates from the eight gelatine agar tubes; label, and incubate at 28° C. (or 30° C.).

10. Liquefy three wort gelatine tubes and to them

add 0.1 c.c. of the diluted milk from capsules I, II, and III respectively.

11. Pour plates from the wort gelatine; label, and incubate at 20° C.

12. Add to each of three agar tubes 0.1 c.c. of the diluted milk from capsule II, III, and IV.

13. Pour plates from the agar tubes; label, and incubate at 37° C.

14. After twenty-four and forty-eight hours' incubation, "count" the agar plates and estimate the number of "organisms growing at 37° C." present per cubic centimetre of the sample of milk.

15. After three, four, or five days' incubation, "count" the gelatine agar plates and estimate therefrom the total number of organisms present per cubic centimetre of the sample of milk.

16. After a similar interval "count" the wort gelatine plates and estimate the number of moulds and yeasts present per cubic centimetre of the sample of milk.

Qualitative.—The qualitative bacteriological examination of milk is chiefly directed to the detection of the presence of one or more of the following pathogenic bacteria:

I. Members of the typhi and coli groups.
II. Bacillus enteritidis of Gaertner.
III. Bacillus enteritidis sporogenes.
IV. Vibrio choleræ.
V. Bacillus diphtheriæ.
VI. Bacillus tuberculosis.
VII. Streptococcus pyogenes longus.
VIII. Staphylococcus pyogenes aureus.

Of these, the first six occur as accidental contaminations (the vehicle of transmission in the case of the first five usually being water), while the last three are usually derived directly from the cow.

In milk, as in water, the first essential is the con-

centration of the bacterial contents of a large volume of the sample into a small compass. In this process, however, thorough centrifugalisation is substituted for filtration.

Apparatus Required:

A centrifugal machine. This machine, to be of real service in the bacteriological examination of milk, must conform to the following requirements:

1. The centrifugal machine must be of such size, and should carry tubes or bottles of such capacity, as to enable from 250 to 500 c.c. of milk to be manipulated at one time.
2. The rate of centrifugalisation should be from 2500 to 3000 revolutions per minute.
3. The portion of the machine destined to carry the tubes should be a metal disc, of sufficient weight to ensure good "flank" movement, continuing over a considerable period of time. In other words, the machine should run down very gradually and slowly after the motive power is removed, thus obviating any disturbance of the relative positions of particulate matter in the solution that is being centrifugalised.
4. The machine should preferably be driven by electricity, or by power, but in the case of hand-driven machines—
   (a) The gearing should be so arranged that the requisite speed is obtained by not more than forty or fifty revolutions of the crank handle per minute, so that it may be maintained for periods of twenty or thirty minutes without undue exertion.
   (b) The handle employed should be provided with a special fastening (*e. g.*, a clutch similar to that employed for the free wheel of a bicycle), or should be readily detachable so that, on ceasing to turn, the handle should not, by its weight and air resistance, act as a brake and stop the machine too suddenly.

One of the few satisfactory machines of this class is shown in figure 169.

Sterile centrifugal tubes, of some 60–70 c.c. capacity, tapering to a point at the closed end, plugged with cotton-wool.

Sterilised cork borer.
Case of sterile pipettes, 10 c.c. (in tenths of a cubic centimetre).
Case of sterile pipettes, 1 c.c. (in tenths of a cubic centimetre).
Flask of sterile bouillon.

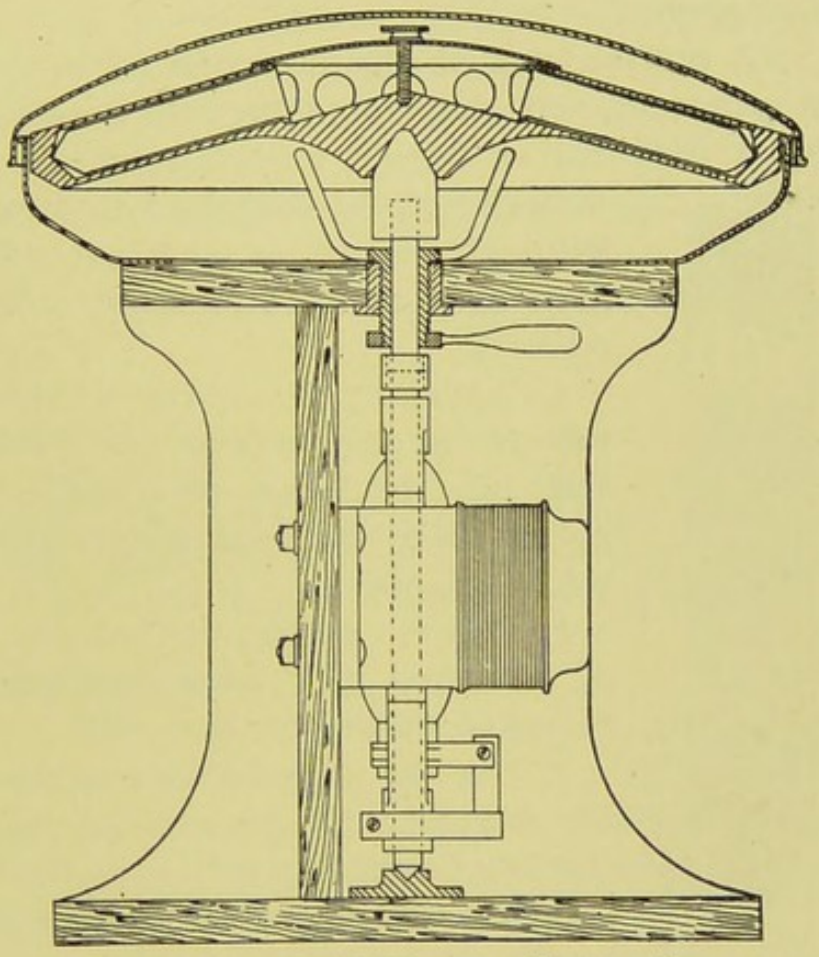


Fig. 169.—Electrically driven centrifugal machine.

METHOD.—

1. Fill the milk sample into the tubes, and replace the cotton-wool plugs by solid rubber stoppers (sterilised by boiling), and fit the tubes in the centrifugal machine.

2. Centrifugalise the milk sample for twenty to thirty minutes at a speed of 2500 revolutions per minute.

3. Remove the motive power and allow the machine to slow down gradually.

4. Remove the tubes of milk from the centrifuge. Each tube will now show (Fig. 170):

(a) A superficial layer of cream (varying in thickness with different samples) churned into a semi-solid mass, which can be shown to contain some organisms and a few leucocytes.

(b) A central layer of separated milk, thin, watery, and opalescent, and containing extremely few bacteria.

(c) A sediment or deposit consisting of the great majority of the contained bacteria and leucocytes, together with adventitious matter, such as dirt, hair, epithelial cells, etc.

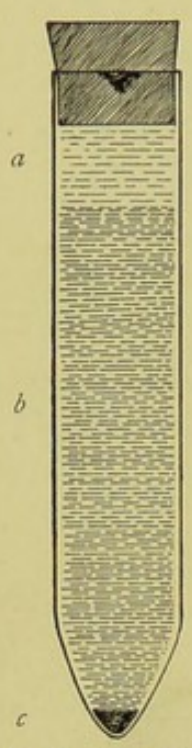


Fig. 170.—Milk in centrifuge tube.

5. Withdraw the rubber stopper and remove a central plug of cream from each tube by means of a sterile cork borer; place these masses of cream in sterile capsules.

6. Remove all but the last 3 or 4 c.c. of separated milk from each tube, by means of sterile pipettes.

7. Mix the deposits thoroughly with the residual milk, pipette the mixture from each tube into a fresh sterile tube, and mix together; then fill with sterile bouillon (or normal saline solution).

8. Place the mixed deposits in the centrifuge, counterpoise with another tube containing an equal volume of water, and centrifugalise, as before.

9. Pipette off all the supernatant fluid and invert the tube to drain on to a pad of sterilised cotton-wool, contained in a beaker. (This wool is subsequently cremated.)

10. Examine both cream and deposit microscopically—

(a) In hanging drops.

(b) In film preparations stained carbolic methylene-

blue, Gram's method, Neisser's method, Ziehl-Neelsen's method.

11. Adapt the final stages of the investigation to the special requirements of each individual sample, as follows:

Members of the Typhoid and Colon Groups.—
Bacillus Enteritidis of Gaertner.—
Bacillus Enteritidis Sporogenes.—
Vibrio Choleræ.—

When searching for any or either of these organisms, emulsify the deposit with 10 c.c. sterile bouillon and proceed with the examination as described under water.

NOTE.—The B. coli communis, derived from the alvine discharges of the cow, is almost universally present in large or small numbers, in retail milk. Its detection, therefore, unless in enormous numbers, when it indicates want of cleanliness, is of little value.

B. Diphtheriæ.—

(A) 1. Plant three sets of serial cultivations from (*a*) cream (twelve tubes in each set) upon oblique inspissated blood-serum, (*b*) deposit (twelve tubes in each set) upon oblique inspissated blood-serum, and incubate at 37° C.

2. Pick off any suspicious colonies which may have made their appearance nine hours after incubation, and subcultivate upon blood-serum; return the original tubes to the incubator.

3. Repeat this after eighteen hours' incubation.

4. From the resulting growths make cover-slip preparations and stain carbolic methylene-blue, Neisser's method, Gram's method.

5. Inoculate guinea-pigs subcutaneously with forty-eight-hour-old glucose bouillon cultivation derived from the first subcultivation, and observe the result.

6. Inoculate guinea-pigs subcutaneously with filtered

glucose bouillon cultivations (toxins ?) and observe the result.

(B) 1. Emulsify the remainder of the deposit with 3 c.c. sterile bouillon and inoculate two guinea-pigs, thus: guinea-pig *a*, subcutaneously with 1 c.c. emulsion; guinea-pig *b*, subcutaneously with 2 c.c. emulsion; and observe the result.

2. If either or both of the inoculated animals succumb, make complete post-mortem examination and endeavour to isolate the pathogenic organisms from the local lesion.

Bacillus Tuberculosis.—Add 5 c.c. sterile bouillon to the deposit in the tube and emulsify thoroughly.

(A) 1. Inoculate each of three guinea-pigs (previously tested with tuberculin, to prove their freedom from spontaneous tuberculosis) subcutaneously at the inner aspect of the bend of the left knee, with 1 c.c. of the emulsion.

2. Introduce a small quantity of the cream into a subcutaneous pocket prepared at the inner aspect of the bend of the right knee of each of these three animals. Place a sealed dressing on the wound.

3. Observe carefully, and weigh accurately each day.

4. Kill one guinea-pig at the end of the second week and make a complete post-mortem examination. (Compare Fig. 158, page 307.)

5. If the result of the examination is negative or inconclusive, kill a second guinea-pig at the end of the third week and examine carefully.

6. If still negative or inconclusive, kill the third guinea-pig at the end of the *sixth* week. Make a careful post-mortem examination.

(B) 1. Place the tube containing the remainder of the emulsion in a water-bath at 56° C. for ten minutes.

2. Remove the tube from the water-bath and cool rapidly.

3. Inoculate each of two guinea-pigs, intraperitoneally, with 1 c.c. of the emulsion.

4. Kill one of the guinea-pigs at the end of the first week and examine carefully.

5. Kill the second guinea-pig at the end of the second week and examine carefully.

NOTE.—No value whatever attaches to the result of a microscopical examination for the presence of the B. tuberculosis unless confirmed by the result of inoculation experiments.

Streptococcus Pyogenes Longus.—

(A) 1. Plant serial cultivations from the deposit upon (*a*) oblique inspissated blood-serum (six tubes in series) and (*b*) oblique nutrient agar (six tubes in series).

2. If the resulting growth shows colonies which resemble those of the streptococcus, make subcultivations upon agar and in bouillon in the first instance and study carefully.

(B) 1. Plant a large loopful of the deposit into each of three tubes of glucose formate bouillon, and incubate anaerobically (in Buchner's tubes) for twenty-four hours at 37° C.

2. If the resulting growth resembles that of the streptococcus, make subcultivations upon nutrient agar.

3. Prepare subcultivations of any suspicious colonies that appear, upon all the ordinary media, and study carefully.

If the streptococcus is successfully isolated, inoculate serum bouillon cultivations into the mouse, guinea-pig, and rabbit, to determine its pathogenicity and virulence.

Staphylococcus Pyogenes Aureus.—

1. Prepare serial cultivations upon oblique nutrient agar (eight tubes in series).

2. Incubate at 37° C. for twenty-four hours.

3. Pick off any suspicious colonies, plant on oblique agar, and incubate at 20° C. Observe pigment formation.

4. Prepare subcultivations from any suspicious growths upon all the ordinary media and study carefully.

ICE CREAM.

Collection of the Sample.—

1. Remove the sample from the drum in the ladle or spoon with which the vendor retails the ice cream, and place it at once in a sterile copper capsule, similar to that employed for earth samples.

2. Pack for transmission in the ice-box.

3. On arrival at the laboratory place the copper capsules containing the ice cream in the incubator at 20° C. for fifteen minutes—that is, until at least some of the ice cream has become liquid.

Qualitative and Quantitative Examination.—Treat the fluid ice cream as milk and conduct the examination in precisely the same manner as described for milk (*vide* page 346).

EXAMINATION OF CREAM AND BUTTER.

Collection of the Sample.—Collect, store, and transmit samples to the laboratory, precisely as is done in the case of ice cream.

Quantitative.—

Apparatus Required:

Sterile test-tube.
Sterilised spatula.
Water-bath regulated at 42° C.
Case of sterile plates.
Case of sterile graduated pipettes, 1 c.c. (in hundredths).
Tubes of gelatine-agar (+10 reaction).
Plate-levelling stand, with its water chamber filled with water at 42° C.

METHOD.—

1. Transfer a few grammes of the sample to a sterile test-tube by means of the sterilised spatula.

2. Place the tube in the water-bath at 42° C. until the contents are liquid.

3. Liquefy eight tubes of gelatine-agar and place them in the water-bath at 42° C., and cool down to that temperature.

4. Inoculate the gelatine-agar tubes with the following quantities of the sample by the help of a sterile pipette graduated to hundredths of a cubic centimetre —viz., 0.1, 0.2, 0.3, 0.5, 0.01, 0.02, 0.03, and 0.05 c.c.

5. Pour a plate cultivation from each of the gelatine-agar tubes and incubate at 28° C.

6. "Count" the plates after three days' incubation, and from the figures thus obtained estimate the number of organisms present per cubic centimetre of the sample.

Qualitative.—

Apparatus Required:

Sterile beaker, its mouth plugged with sterile cotton-wool.
Scales and weights.
Sterilised spatula.
Water-bath regulated at 42° C.
Separatory funnel, 250 c.c. capacity, its delivery tube protected against contamination by passing it through a cotton-wool plug into the interior of a small Erlenmeyer flask which serves to support the funnel. This piece of apparatus is sterilised *en masse* in the hot-air oven.
Centrifugal machine.
Sterile tubes (for the centrifuge) closed with solid rubber stoppers.
Case of sterile pipettes.

METHOD.—

1. Weigh out 100 grammes of the sample in a sterile beaker.

2. Plug the mouth of the beaker with sterile cotton-wool and immerse the beaker in a water-bath at 42° C. until the contents are completely liquefied.

3. Fill the liquefied butter into the sterile separatory funnel.

4. Transfer the funnel to the incubator at 37° C. and allow it to remain there for four days.

At the end of this time the contents of the funnel will have separated into two distinct strata.

(a) A superficial oily layer, practically free from bacteria.

(b) A deep watery layer, turbid and cloudy from the growth of bacteria.

5. Draw off the subnatant turbid layer into sterile centrifugal tubes, previously warmed to about 42° C., and centrifugalise at once.

6. Pipette off the supernatant fluid and fill the tubes with sterile 1 per cent. sodium carbonate solution previously warmed slightly; stopper the tubes and shake vigourously for a few minutes.

7. Centrifugalise again.

8. Pipette off the supernatant fluid; filling the tubes with sterile bouillon, shake well, and again centrifugalise, to wash the deposit.

9. Pipette off the supernatant fluid.

10. Prepare cover-slip preparations, fix and clear as for milk preparations, stain carbolic methylene-blue, Gram's method, Ziehl-Neelsen's method, and examine microscopically with a $\frac{1}{12}$-inch oil-immersion lens.

11. Proceed with the examination of the deposit as in the case of milk.

EXAMINATION OF UNSOUND MEATS.

(Including Tinned or Potted Meats, Fish, Etc.)

Qualitative.—

Apparatus Required:

Erlenmeyer flask (500 c.c. capacity) containing 250 c.c. sterile bouillon and fitted with solid rubber stopper.

Scissors and forceps.

Water steriliser.
Hypodermic syringe.
Case of sterile capsules.
Filtering apparatus as for water analysis.
Case of sterile plates.
Case of sterile graduated pipettes, 10 c.c. (in tenths of a cubic centimetre).
Case of sterile graduated pipettes, 1 c.c. (in tenths of a cubic centimetre).
Plate-levelling stand.
Tubes of nutrient gelatine.
Tubes of nutrient agar.
Water-bath regulated at 42° C.
Bulloch's apparatus.

METHOD.—

(A) 1. Mince a portion of the sample by the aid of sterile scissors and forceps, and add the minced sample to the bouillon in the flask.

2. Make an extract by standing the flask in the water-bath at 42° C. for half an hour, shaking its contents from time to time.

3. Pipette off 10 c.c. of the extract into a sterile test-tube and remove for use under section C.

4. Filter the extract through a sterile Berkfeld filter.

5. Emulsify the bacterial residue with 10 c.c. sterile bouillon.

6. Pour a set of gelatine and a set of agar plates from tubes containing 0.2, 0.3, and 0.5 c.c. of the extract, and incubate aerobically, the gelatine set at 20° C., the agar at 37° C.

7. Pour duplicate sets of gelatine and agar plates and incubate anaerobically in Bulloch's apparatus, at similar temperatures.

8. Subcultivate from the colonies that make their appearance and identify the various organisms.

9. Continue the investigations with reference to the detection of pathogenic organisms as described under water (page 327 *et seq.*).

(B) 1. Feed rats and mice on portions of the sample and observe the result.

2. If any of the animals die, make complete post-mortem examinations and endeavour to isolate the pathogenic organisms.

(C) 1. Inoculate rats, mice, and guinea-pigs subcutaneously and intraperitoneally with various quantities of the bouillon extract, and observe the result.

2. If any of the animals succumb to the inoculation, make careful post-mortem examinations and endeavour to isolate the pathogenic organisms.

EXAMINATION OF FILTERS.

Porcelain filter candles are examined with reference to their power of holding back *all* the micro-organisms suspended in the fluids which are filtered through them, and permitting the passage of only germ-free filtrates. The examination is conducted as follows:

Apparatus Required:

Filtering apparatus—one or other of those described under Examination of Water. The actual filter candle that is used must be the one it is intended to test; the arrangement of the apparatus will therefore need to be varied with each different form of filter (see also pages 47, 48).
Plate-levelling stand.
Case of sterile plates.
Case of sterile pipettes, 10 c.c. (in tenths).
Case of sterile pipettes, 1 c.c. (in tenths).
Tubes of nutrient gelatine.
Flask containing sterile normal saline solution.
Sterile measuring flask, 1000 c.c. capacity.

METHOD.—

1. Prepare surface cultivations, on nutrient agar in a culture bottle, of the Bacillus prodigiosus, and incubate at 20° C., for forty-eight hours.

2. Pipette 5 c.c. sterile normal saline into the culture bottle and emulsify the entire surface growth in it.

3. Pipette the emulsion into the sterile measuring

flask and dilute up to 1000 c.c. by the addition of sterile water.

4. Pour the emulsion into the filter reservoir and start the filtration.

5. When the filtration is completed, pour six gelatine plates each containing 1 c.c. of the filtrate.

6. Incubate at 20° C. until, if necessary, the completion of seven days.

EXAMINATION OF DISINFECTANTS.

Disinfectants or Germicides are examined with reference to three points:

(A) **Inhibition coefficient**—*i. e.*, that percentage of the disinfectant present in the nutrient medium which is sufficient to prevent the growth and multiplication of bacteria therein.

(B) **Inferior lethal coefficient**—*i. e.*, the time exposure necessary to kill vegetative forms suspended in water at 20° to 25° C., in which the disinfectant is present in *medium* concentration (concentration insufficient to cause plasmolysis).

(C) **Superior lethal coefficient**—*i. e.*, the time exposure necessary to kill spores under conditions similar to those obtaining in B.

The methods here detailed only specifically refer to those disinfectants mentioned under Germicides in the Scheme for the Study of Bacteria (page 252), but the technique is practically similar for all other chemical disinfectants.

Inhibition Coefficient.—

Apparatus Required:

Case of sterile pipettes, 10 c.c. (in tenths).
Case of sterile pipettes, 1 c.c. (in tenths).
Sterile tubes or capsules for dilutions.
Tubes of nutrient bouillon.

Materials Required:

1. Five per cent. aqueous solution of carbolic acid.

2. One per cent. aqueous solution of perchloride of mercury.
3. One-tenth per cent. aqueous solution of formaldehyde.

METHOD.—

1. Prepare a series of six tube cultivations, in bouillon (each tube containing 10 c.c. of medium), of each organism employed in the test and add 2 c.c. of the 5 per cent. carbolic acid solution (1 : 100) to the first, 1 c.c. (1 : 200) to the second, 0.6 c.c. (1 : 300) to the third, 0.5 c.c. (1 : 400), to the fourth, 0.4 c.c. (1 : 500) to the fifth, and 0.2 c.c. (1 : 1000) to the sixth.

2. Prepare a similar series of tube cultivations and add 0.1 c.c. (1 : 1000), 0.05 c.c. (1 : 2000), 0.03 c.c. (1 : 3000), 0.025 c.c. (1 : 4500), 0.02 c.c. (1 : 5000), and 0.01 c.c. (1 : 10,000) of the 1 per cent. perchloride of mercury solution.

3. Prepare a similar series of tube cultivations and add 1 c.c. (1 : 1000), 0.4 c.c. (1 : 2500), 0.2 c.c. (1 : 5000), 0.1 c.c. (1 : 10,000), 0.075 c.c. (1 : 15,000), and 0.05 c.c. (1 : 20,000) of the 0.1 per cent. formaldehyde solution.

4. Incubate all three sets of cultivations under optimum conditions as to temperature and atmosphere.

5. Examine each of the culture tubes from day to day, until the completion of seven days, and note those tubes, if any, in which growth takes place.

Inferior Lethal Coefficient.—

Apparatus Required:

Highly concentrated solutions of the disinfectants.
Sterile test-tubes in which to make dilutions from the concentrated solutions of the disinfectants.
Hanging-drop slides.
Cover-slips.
Erlenmeyer flask containing 100 c.c. sterile distilled water.
Case of sterile pipettes, 10 c.c. (in tenths of a cubic centimetre).
Case of sterile pipettes, 1 c.c. (in tenths of a cubic centimetre).

METHOD.—

1. Prepare a surface cultivation of each of the "test" organisms upon nutrient agar in a culture bottle and incubate under optimum conditions for forty-eight hours; then examine the cultivation microscopically to determine the absence of spores.

2. Prepare solutions of different percentages of each disinfectant.

3. Make a series of hanging-drop preparations from the agar culture, using a loopful of disinfectant solution of the different percentages to prepare the emulsion on each cover-slip.

4. Examine microscopically and note the strongest solution which does not cause plasmolysis and the weakest solution which does plasmolyse the organism.

5. Make control preparations of these two solutions and determine the percentage to be tested.

6. Pipette 10 c.c. sterile water into the culture bottle and suspend the entire surface growth in it.

7. Transfer the suspension to the Erlenmeyer flask and mix it with the 90 c.c. of sterile water remaining in the flask.

8. Pipette 10 c.c. of the diluted suspension into each of ten sterile test-tubes.

9. Label one of the tubes "Control" and place it in the incubator at 20° C.

10. Add to each of the remaining tubes a sufficient quantity of a concentrated solution of the disinfectant to produce the percentage previously determined upon (*vide* step 5).

11. Incubate the tubes at 20° C.

12. At hourly intervals remove the control tube and one of the tubes with added disinfectant from the incubator.

13. Make a subcultivation from both the control and the test suspension, upon the surface of nutrient agar; incubate under optimum conditions.

14. Observe these culture tubes from day to day until the completion of seven days, and determine the shortest exposure necessary to cause the death of vegetative forms.

Superior Lethal Coefficient.—

1. Prepare surface cultivations of the "test" organisms upon nutrient agar in a culture bottle, and incubate under optimum conditions, previously determined, for the formation of their spores.

2. Employ that percentage solution of the disinfectant determined in the previous experiment, and complete the investigations as detailed therein, steps 6 to 14, increasing the interval between planting the subcultivations to two, three, or five hours if considered advisable.

NOTE.—Where it is necessary to leave the organisms in contact with a strong solution of the disinfectant for lengthy periods, some means must be adopted to remove every trace of the disinfectant from the bacteria before transferring them to fresh culture media; otherwise, although not actually killed, the presence of the disinfectant may prevent their development, and so give rise to an erroneous conclusion. In such cases proceed as follows:

1. Transfer the suspension of bacteria to sterile centrifugal tubes; add the required amount of disinfectant, and allow it to remain in contact with the bacteria for the necessary period.

2. Centrifugalise thoroughly, pipette off the supernatant fluid; fill the tube with sterile water and distribute the deposit evenly throughout the fluid.

3. Centrifugalise again, pipette off the supernatant fluid; fill the tube with sterile water; distribute the deposit evenly throughout the fluid, and transfer the suspension to a litre flask.

4. Make up to a litre by the addition of sterile water; filter the suspension through a sterile porcelain candle.

5. Emulsify the bacterial residue with 5 c.c. sterile bouillon.

6. Prepare the necessary subcultivations from this emulsion.

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