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A LABORATORY HANDBOOK OF BACTERIOLOGY

RUDOLF ABEL
M. H. GORDON

SECOND EDITION

OXFORD
MEDICAL PUBLICATIONS

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TRANSLATOR'S PREFACE

TO THE

SECOND ENGLISH EDITION

Since the first English edition of Professor Abel's book was issued, the German original has gone through five more editions. I have much pleasure in acknowledging the assistance which has been rendered by Dr. P. Hamill in preparing the present edition.

M. H. G.

London,
December, 1912.

PREFACE

TO THE

FIRST ENGLISH TRANSLATION

ANY one who has been at all in the position to follow for some time the evolution of medicine and natural science cannot fail to have noticed how the importance and the use of bacteriological knowledge has constantly been spreading. For the physician and the veterinary surgeon bacteriology is an indispensable aid towards the recognition of infectious diseases in men and animals. Only by the aid of a certain amount of bacteriological knowledge is he enabled to understand and apply the successful methods of serumtherapeutics, of protective inoculation, of the delicate modern serodiagnostic methods of investigation, which promise still greater results. The commissioned medical officer has absolute need of bacteriology in order to employ proper and efficient measures to ward off pestilence and to prevent the spread of infectious diseases. Indeed, one may say that without bacteriology hygiene would never have attained its present height. The chemist, the druggist, the engineer, and even many commercial men, for instance, those working in the production of beer and wine, in the industry of preserves and of foodstuffs, require a certain amount of knowledge of bacteriology for their business. Thus it has become the subject of study in ever-increasing circles.

In writing the present pocket edition it has been the wish of the author to provide those studying bacteriology with a guide to practical laboratory work. It is not meant to take the

place of a text-book or of personal instruction, but to serve as a supplement to the above, to give practical hints, and to collect in practical form those technical details which so easily slip the memory.

Its popularity in Germany, where since 1898 an almost yearly edition has been necessary, is due above all to its efforts to be clear and concise, and to bring forward only reliable and fully proved methods of examination, which are as simple as possible to undertake and to put into practice. Especially intended in the first place for the use of the physician and the veterinary surgeon, it will also help the apothecary and the chemist and those of other callings, as it also includes what is necessary for their requirements.

Through the kindness of the English publishers of this little book, I have been able to look through the translation. I can only express my full approval of the translation, and will only say that the additions in the English edition made to bring in special methods customary in England seem to me to add more value to its contents.

I hope with all my heart that this little book may be of use to the English student and friend of bacteriology, just as it has been able to win for itself a circle of friends in Germany, and that it may assist in spreading and deepening the knowledge of bacteriology for the benefit of the various callings in need of it, and so *pro bono publico*.

R. ABEL.

BERLIN,
August, 1907.

TRANSLATOR'S PREFACE

In rendering Professor Abel's Bacteriological Pocket Book available for English readers, the opportunity has been taken of including some of the more important methods introduced in the course of recent development of Bacteriology in this country.

Dr. Houston has kindly taken charge of the section dealing with the bacteriological examination of water, milk, sewage, &c., and Dr. Horder has been so good as to revise the section on methods of removing material from the body for bacteriological examination. The bacteriological examination of dust and air has been dealt with in somewhat more detail than in the original; and a section has been added on methods of examining the blood in relation to immunity. Minor additions inserted elsewhere are enclosed in brackets.

The translator desires to record his thanks to Col. Leishman for kindly supplying the description of his methods of estimating phagocytosis, and of determining the presence of stimulins; and he also wishes to acknowledge his indebtedness to Dr. Louis Elkind for assistance in the work of translation.

M. H. G.

London,
August, 1907.

CONTENTS

REFERENCES

A. I. P.	Annales de l'Institut Pasteur.
B. Klin. W.	Berliner Klinische Wochenschrift.
B. M. J.	British Medical Journal.
Centr. f. Bakt.	Centralblatt für Bakteriologie (Or. = Abt. I, Original-Band).
D. m. W.	Deutsche medizinische Wochenschrift.
Hyg. Rdsch.	Hygienische Rundschau.
L. C. C.	Report to the London County Council.
L. G. B.	Report of the Medical Officer, Local Government Board.
M. m. W.	Münchener medizinische Wochenschrift.
Phil. Trans.	Philosophical Transactions of the Royal Society.
P. R. S.	Proceedings of the Royal Society.
R. S. C.	Report of the Royal Commission on Sewage Disposal.
Zsch. f. Hyg.	Zeitschrift für Hygiene.

Further references in the text are given with sufficient fullness to directly identify their origin.

I

The Microscope

In bacteriological work dry lenses are used for examining microscopical preparations and colonies of bacteria. For observation of the bacteria themselves an immersion lens is necessary.

When examining a preparation with the *immersion lens* a drop of the immersion fluid is first transferred to the clean (for method of cleansing see p. 8) surface of the cover-glass (usually squares 18 mm. long and 0·16 mm. thick) by means of a glass rod. As a rule the immersion fluid employed is cedar oil (thickened cedar oil, not the thinner sort used for clearing sections). The tube of the microscope is lowered until the objective just touches the drop of immersion fluid. At this point one looks through the microscope and continues to lower the tube by means of the coarse adjustment until an indistinct picture of the object comes into view. Focussing is then completed by means of the fine adjustment.

Bacteria are examined (*a*) in unstained and (*b*) stained preparations.

(*a*) *Unstained preparations* serve for the purpose of studying the characteristics of living micro-organisms, and are made in the form of *hanging drop preparations* in the following way (for dark ground

illumination, &c., see p. 6). On the centre of a clean cover-glass which is placed either on the laboratory bench or held with a Cornet's forceps, one puts by means of a platinum loop which has been thoroughly sterilized in the flame a drop either of physiological salt solution (0·7%–0·8%) or of broth, or of peptone water (see pp. 13, 23). By means of a sterilized platinum needle a trace (not too much) of the material which contains bacteria is next transferred to this drop on the cover-glass. In cases where the fluid to be examined is not too rich in bacteria, a drop of it may be placed on the cover-glass without dilution. A hanging drop slide is now taken, and the margin of its cavity covered with vaseline. The cover-glass is then placed upon this cavity of the slide in such fashion that the drop lies freely in the hollow. The cover-glass must be firmly pressed down all round on the vaseline so that the drop hangs in a completely closed space; for if this is not the case evaporation occurs, and not only does the drop tend to dry up, but currents are apt to develop in it which drag along the bacteria and thus produce movements which may be mistaken for true motility.

Examination by the hanging drop method has the advantage over the other method of examining fluid directly, e.g. by spreading the material between a cover-glass and an ordinary slide, in that evaporation is more readily prevented, and the material is less liable to escape and infect instruments, &c.

When examining a hanging drop preparation microscopically, in the first place a low power is used in conjunction with a narrow diaphragm, and the

preparation is placed on the stage of the microscope in such fashion that the edge of the drop passes directly across the centre of the field of vision. If the low power is replaced by an immersion lens, and the lenses are correctly centred, the edge of the drop will then again pass across the centre of the field; that is to say, it will be readily perceptible. When replacing the low power objective with the immersion lens, therefore, one places a drop of cedar oil on the cover-glass without moving the preparation, widens the diaphragm and lowers the tube until the lens touches the oil, then looks through the microscope and lowers the tube further, using the greatest care, and proceeds slowly downwards with the fine adjustment until the margin of the drop comes into view. It is advisable to move the slide very gently from side to side with the hand, for in this way one is able to perceive at once if the lens has been lowered too far, and by pressing upon the cover-glass threatens to break it. Further, the margin of the drop which in the first instance appears as a shadow like any other object is more easily seen in a state of movement than of rest. Outside the edge of the drop one may observe a fine network due to precipitates resulting from evaporation on the under surface of the cover-glass round the drop.

Experienced observers sometimes examine the drop by an immersion lens straight away; beginners, however, may add to it a trace of very dilute fuchsin solution for the purpose of enabling them to find the drop more easily. If in small quantity, the dye does not interfere with the bacteria. In any case, the edge

of the drop is looked for because its outline is more easily perceived than the rest of the drop. In consequence of the more active gas exchange between air and fluid which prevails at the edge of the drop, motile bacteria especially tend to collect round the margin. Distilled water should not be used for hanging drop preparations because it tends to reduce motility. Cultures which have just been taken from the incubator at 37° C. should be examined in a fluid previously warmed, so that the bacteria do not appear non-motile in consequence of rigidity due to cold.

Try to avoid mistaking molecular or Brownian movement, i.e. vibration from side to side, for true motility or change of position. The drop should be as flat as possible in order that it may be accessible in all its parts to the immersion lens, for if the drop is too large, the deeper parts lie external to the focal range of the lens.

If the cover-glass is sterilized in the flame and a droplet of transparent nutrient medium, either liquid or rendered fluid, is placed upon it, a culture preparation is obtained in which the growth of bacteria can be directly followed with the eye. The warm stage is of use in this connexion.

As soon as the examination of a hanging drop preparation is finished, the cover-glass is raised from the glass slide in such manner that the drop does not touch the slide, and the cover-glass with its attached drop is plunged into strong sulphuric acid for the purpose of destroying the bacteria. The glass slide may then be used for another specimen. At the end of the examination the vaseline is removed from the

slide by means of blotting-paper, and the slide subsequently cleaned with a rag steeped in benzene.

(*b*) *Stained preparations.* As regards the preparation and staining of film-preparations and sections see pp. 56, 60.

Illumination. In examining the hanging drop, it is well to employ the concave mirror, and either a narrow stop or a contracted iris diaphragm, in order that a contour or refraction image may be obtained. The more powerful the objective, the wider should the diaphragm be opened—it should be about half open if the immersion lens is used. The Abbe condenser is put out of action by application of the diaphragm, so that it is not necessary to remove it. When examining stained preparations, the plane mirror is used without the diaphragm and an absorption or colour image is obtained: in the last case the *Abbe condenser* comes into action, and the latter should be adjusted in such a way that the light refracted by it is thrown directly upon the spot where the specimen to be examined lies. First the specimen is placed in position, under a low power. Then the plane mirror is turned towards an object which lies at some distance—a tree or a house, for instance—and the condenser is raised or lowered without further moving the tube until one perceives in and upon the image of the specimen a distinct figure of the reflected object. The condenser is left in this position, and now the mirror is turned from the tree or house to any distant source of light which does not yield a reflection of its own, e.g. white sky. The adjustment of the Abbe condenser in the particular way described, however,

is not always necessary. In case of a distant light source it is as a rule sufficient to screw this apparatus up as high as possible; but when the source of light is near at hand the condenser ought to be lowered if one wishes to obtain a good effect. If the light is bad, the condenser should be minutely adjusted.

Glaring sky light is relieved by interposing a plane of blue glass in a slot below the stage of the microscope, or by means of blinds to the windows.

As regards artificial light, incandescent light is especially suitable. An oil lamp may also be used provided that its light is made to pass through a blue glass disk in the diaphragm of the microscope, or through a shoemaker's ball, or through a bulb filled with copper and ammonium sulphate. The last solution is made up by adding ammonia to an intensely blue solution of copper sulphate until the light which passes through appears purely white in the microscope. Electric light requires to be relieved by interposing a blue or ground glass screen in the microscope, or by application of a 'frosted pear'. In case of the source of light being near at hand and its reflection interfering with the examination of specimens, one should use the concave mirror, or slightly raise the condenser.

Dark Ground Illumination. This method enables one to examine the finest particles illuminated against a dark background; it is particularly valuable in searching for living spirochaetes of Syphilis, and can also be used for the observation of flagella of living bacteria. Instead of a special microscope (ultramicroscope) one uses for this purpose the reflecting

condenser (Reichert, Leitz) or the paraboloid (Zeiss, Watson). The source of light must be powerful, preferably a Nernst or small arc lamp.

Bacteria, &c., also appear illuminated on a dark ground in drops of Indian-ink suspension prepared after Burri's method (see p. 38).

Directions for cleaning the Microscope.

When removing an immersion specimen from beneath the microscope, one first raises the tube so that the lens is not injured in the process of removing the preparation. On completion of the microscopical examination, the immersion fluid should be removed from the lens by means of fine blotting-paper (Joseph paper); then the lens should be cleansed with a piece of clean wash-leather. If the lens is covered with resinous oil, Canada balsam, or similar material, it may be cleaned with a small piece of leather, or fine blotting-paper wetted either with xylol, benzene, or similar solvent. Care, however, should be taken, for such solvents are apt to dissolve the material with which the lens is cemented.

After use, one protects the microscope against light and dust by placing it either under a dark glass bell jar, or in its box or case. If the objective is not removed, a little piece of fine clean blotting-paper should be placed between the Abbe condenser and the objective to prevent their coming in contact.

For preservation of preparations see pp. 59, 60, 65.

Directions for cleaning Cover-glasses.

1. New cover-glasses are cleaned with alcohol and ether equal parts, or with xylol, or benzene; and a fine linen cloth or thin blotting-paper is used for this purpose. A drop of water must distribute evenly over the surface of the cleaned cover-glasses; and if they fail to pass this test, the cleansing process must be renewed, or the cover-glasses treated in the way directed for old ones. Cover-glasses that have stuck together may be heated on a tin plate over the flame.

Careful burning in the flame frequently facilitates the cleansing process and sometimes is all that is necessary.

2. *Old cover-glasses.* Method (*a*). After use, cover-glasses are immersed in a glass jar containing raw sulphuric acid. For the purpose of cleaning, they are poured with the acid into a white porcelain bowl, and after the acid has been poured off, they are thoroughly washed in running water and then boiled in strong caustic soda or potash. On removal from the boiling alkali they are again washed in water, and subsequently treated with equal parts alcohol and ether, or with xylol or benzene, and cleansed with a piece of linen or thin blotting-paper. The test of cleanliness which all these cover-glasses must pass is that a drop of water placed upon their surface must distribute evenly, and if they fail to pass this test, the process of boiling with caustic potash must be repeated. Method (*b*). According to Zettnow cover-glasses may be cleaned as follows:—(1) They are boiled for ten minutes, stirring the while, in the following

solution :—potassium bichromate 200 grams, hot water 2 litres, strong sulphuric acid 200 c.c. (2) The fluid is poured off and the cover-glasses are rinsed for five minutes in a dilute solution of caustic soda. Both processes are now repeated, the first being continued for only five minutes. The cover-glasses are then washed in water, next in alcohol, and finally cleansed by hand.

II

Sterilization and Disinfection

ALL utensils and nutrient media used for cultivation of micro-organisms must be free of germs: otherwise such germs may develop as contaminations.

The following general rules should be observed : —

1. Minor instruments such as platinum needles, knives, scissors, forceps, glass rods, may be sterilized in the flame. Platinum instruments are heated to redness, but the other instruments need only be heated in such wise that all parts destined to come in contact with the material which contains bacteria are licked over by the flame for at least two seconds. Instruments of metal tend to suffer through heating and to become blunted; the better ones therefore may be sterilized according to the rules laid down in 2 and 3. Only platinum-iridium instruments preserve their edge despite frequent heating; these are useful for subculturing hard or tough cultures (actinomyces or moulds) and also for obtaining blood. Glass plates, smaller glass bowls, and pipettes may also be

sterilized if necessary in the flame. Test-tubes, again, may be sterilized in the same way, and should be heated until their protecting plugs of wool are browned. Parts of syringes, if they are not composed of materials liable to be injured by the flame, may also be sterilized in the same fashion.

2. All other major instruments which can stand dry heat, viz. :— glass utensils, metal instruments which are not soldered, wadding, blotting-paper, &c., are sterilized in the hot air sterilizer by being kept for half an hour at 150° C. and over. Wadding should not be heated above 180° C., since it becomes brown and brittle above that temperature. The same applies to blotting-paper, which also becomes brown. Glass and metal articles may be exposed to 200° C. and higher.

3. Materials that do not stand dry heat, but can be boiled without undergoing any change, are sterilized by being heated in steam. To this category belong all the nutrient media (excepting those which contain coagulable albumen), fluids, rubber goods. In most cases exposure for a quarter to half an hour in flowing steam at 100° C. is sufficient. Larger quantities of fluid must be subjected to longer treatment than smaller (up to one hour), because it takes longer to heat them up to 100° C.: thus litre flasks of fluid require to be boiled for a longer time than test-tubes.

Nutrient media containing spores are sterilized by one of the two following ways :—Either the medium is placed on three successive days for a quarter to one hour in the steamer, and kept between whiles at a temperature of 20° or 37° C., in which case the spores that

escape the first steaming develop, and the bacilli thus formed are destroyed by the subsequent steaming; or else the medium is sterilized by placing it in the autoclave at a temperature of 130° C., and corresponding pressure. This last method has the advantage that one has not got to wait several days until the sterilization is complete, but in the case of gelatine, it is only to be used with care, because this material is apt to become permanently liquefied if autoclaved. In the case of milk and other media containing sugar this method is not applicable, as the medium becomes browned. See individual media. A temperature of 120° C. must be maintained for fifteen minutes to destroy all germs, but at 130° C. they perish within one minute.

Surgical instruments are sterilized by boiling them for fifteen minutes in a 1–5 % solution of soda.

4. Objects or materials which do not withstand either dry heat or boiling without undergoing change, e. g. serum, egg albumen, &c., should either:— (*a*) be collected with absolute asepsis (see pp. 24, 25), or (*b*) should be subjected to fractional sterilization at from 56° to 60° C. daily for from 1 to 4 hours for several (up to eight) consecutive days, and should meanwhile be kept at a temperature of 20° or 37° C. This method, however, cannot be relied upon always, for in the case of some micro-organisms a temperature of about 60° C. only represents the temperature of maximum development, and, further, not all the spores may develop into bacteria between the heatings, or, on the other hand, even new spores may be formed.

(*c*) Such substances may be freed from bacteria by

filtering them through the Pasteur Chamberland, or through the Berkefeld, Pukall, or Isny filter, or an asbestos filter, for the preparation of which see *Centr. f. Bakt.* I, Ref. Bd. 38, Beiheft S. 52, with the aid of an air-pump worked by a jet of water. Albuminous fluids are filtered with difficulty as a rule, but come through easier if slightly warmed. This method can be employed with advantage for the purpose of separating the products of metabolism of bacteria from the bodies of such bacteria. For further details as to the sterilization of serum, see serum preparation, p. 23.

5. Washing and rinsing with 5 per 1,000 corrosive sublimate, then with equal parts alcohol and ether, is for some objects a satisfactory method of sterilization (e.g. for rubber materials, glass dishes, and pipettes), though it does not always succeed. When it is possible, one should destroy the last residue of alcohol and ether in the flame.

6. Where it is only a question of destroying bacteria, e.g. to disinfect used test-tubes, it is best to employ a 1 per 1,000 solution of corrosive sublimate with the addition of 1% HCl or NaCl. Culture vessels which have been used may be freed of living pathogenic germs by boiling them for 1–2 hours in a pot of water, or by placing them in the steamer for this time. Hands infected with bacteria should be thoroughly cleansed as follows:—Rinse them in 1 : 1000 solution of sublimate and salt, or in Cresol soap solution 1 : 100, next wash and scrub them in soap and water, brush them in alcohol, and then in fresh disinfectant solution and wash again. N.B. The first water used for washing, and the brush, may,

under certain circumstances, contain living germs, and should therefore be disinfected if possible. For mode of disinfecting the bodies of infected animals see p. 213.

III

Nutrient Media

THE basis of media most generally used for the cultivation of bacteria is :—

Meat Extract, or Fleischwasser.

1. 500 grams of finely-chopped beef or horseflesh, as free as possible from fat, are warmed in the pot with a litre of ordinary water at 50° C. for half an hour and then boiled from $\frac{1}{2}$–$\frac{3}{4}$ of an hour. See also p. 17 under 1.

2. The fluid is filtered or strained off from the meat, enough water is added to make it up to one litre, and it is then poured into a flask the mouth of which is plugged with wool. From this meat extract nutrient broth, nutrient gelatine, and nutrient agar are made. If it is to be preserved, the meat extract must be heated on three consecutive days for half an hour at a time in the steamer; or it may be sterilized at once by exposing it in the autoclave for fifteen minutes to 120° C.

Broth.

1. To meat extract prepared as above are added peptone (Witte-Rostock or Chapoteaut Paris) 1% or even up to 5%, and $\frac{1}{2}$% of common salt. For some

bacteria the addition of from ¼–1% of grape sugar is advisable, in which case the sugar should be added at the end of the process, because, when boiled for some time, sugar is converted into caramel, which produces brown discoloration of the medium. When the broth has been prepared, the desired amount of sugar should be added from a sterilized 10% solution of it.

2. Heat in the steamer until dissolved.

3. Neutralize either with saturated, with 10%, or with normal solution of sodium carbonate, or with disodium phosphate, or with 25% NaOH. The correct reaction is reached at the point when blue litmus-paper is no longer reddened when a drop of the medium is placed upon it. Red litmus-paper will then be rendered blue. For purpose of comparison, observe the tint produced by a drop of water from the tap. If too much alkalinity has been produced, this may be reduced by adding lactic acid, phosphoric acid, or HCl drop by drop. A small excess of alkali (Na_2CO_3, not NaOH) does, as a rule, no harm (see gelatine, pp. 16, 17).

For neutralization with phenolphthalein as indicator see p. 20.

4. Heat in steamer for ¼–½ hour.

5. Filter. Re-test reaction of the medium, and if necessary correct it. Again steam and filter. Should the filtrate on cooling not be clear, the process of filtration must be repeated, or clear as directed for gelatine (see below).

6. Sterilize in a flask, or else place in tubes and steam on three consecutive days for ¼–½ hour, or autoclave it as directed on p. 13.

Gelatine.

1. To meat extract the same addition is made as in preparation of nutrient broth, together with 10%, or in summer 15%, of the finest white gelatine (SO_2 free).

2-5 are carried out in the same fashion as in the case of nutrient broth. If, despite of a correct reaction and thick filter, one does not obtain a fairly or completely clear filtrate, then the white of an egg or 10-20 c.c. of meat juice expressed in the cold from raw meat may be added to the gelatine after cooling it down to about 50° C. Shake well and filter again after careful boiling.

6. Carry out as in the case of broth.

The gelatine remains firm at temperatures between 20-27° C. (see below): at slightly higher temperatures it melts slowly, but at 35° C. it melts rapidly; when in a melted condition the gelatine solidifies again if cooled below 20° C. Gelatine must not be boiled too frequently, or too long, and care is especially necessary when exposing it to a temperature above 100° C. in the autoclave; for though it stands being heated once to 110° C. for fifteen minutes, its capacity for solidifying is apt to be impaired by exposure to such temperatures. The melting-point of gelatine, in fact, remains the higher the less it is heated. The melting-point of recently prepared gelatine is always somewhat lower than that of gelatine that has been left in a solidified condition for twenty-four hours or longer.

Nutrient gelatine with a specially high melting-point prepared according to Forster. In a small vessel

containing 1 litre of sterile broth and warmed to 60° C., dissolve 100–150 grams of gelatine. Add KOH to reduce the acidity till it is faint, then make slightly alkaline with Na_2CO_3. Now add the white of an egg. Stand the vessel in a large boiling-pot filled with boiling water and stir the gelatine well. In three minutes it becomes heated up to 98–99° C. Now close the cover of the large boiling-pot, and boil it together with the vessel containing the gelatine for about fifteen minutes. Filter through a warm-water funnel at a temperature of 60° C. (the filter, funnel, and collecting flask having been previously sterilized). Collect the whole of the gelatine in one flask. Transfer it aseptically into sterile test-tubes and heat for twenty minutes in the steamer, then cool by immersion in cold water. The gelatine is now ready for use, and sterile; its melting-point lies after several days between 29–30° C.: but at first is somewhat lower. The principal point of this method is the brief duration of the boiling of the gelatine.

For certain purposes it is well to make the gelatine somewhat alkaline. An addition of 10–15 c.c. of normal soda solution per litre of gelatine over the neutral point scarcely ever does any harm. Certain kinds of bacteria require a still higher alkalinity (see later). The following soda solutions are of equivalent value:—10 c.c. of normal solution of soda, 5·3 c.c. of 10% solution of water-free soda, or 14·3 c.c. of 10% solution of crystallized soda. A 10% solution of crystallized soda contains 3·7% Na_2CO_3. Normal soda solution contains 5·3% Na_2CO_3.

[The following is a convenient and quick way of

making gelatine. *Composition of the Medium* : Gelatine (yellow gold label) 100 grams (in summer 120), Peptone (Witte) 10 grams, Sodium Chloride 5 grams, Meat Extract to 1 litre. *Directions:*—Put 500 c.c. of the meat extract into a white enamelled saucepan, heat to about 70° C., add peptone, and stir with a small wooden spoon. When the peptone has dissolved, add the gelatine, and stir again till dissolved. Now add remainder of the broth, thoroughly mix, make alkaline with Liq. potassi (till blue litmus-paper is rendered slightly more blue), add the beaten white of one egg, put in a flask and heat in the steamer for 30 minutes. Filter through two thicknesses of Swedish or Rhenish filter-paper moistened with sterile distilled water (placing the funnel in warm filter jacket) into a sterile flask, put into tubes, and sterilize in steamer for 30 minutes on two successive days.]

Agar.

1. To meat extract the same addition is made as in the preparation of nutrient broth, and also $1\frac{1}{2}$–2 % of finely chopped or powdered agar. It is well to add the agar to the meat extract a couple of hours before the other ingredients, for in this way the agar becomes softened, and dissolves more easily.

2–4 are carried out as in the case of nutrient broth. Now test the reaction of the medium and correct it if necessary.

5. The turbid flocculent medium must next be filtered. This is not easily effected through filter-paper, even with the aid of a warm funnel, or in the steamer. Therefore it is better to filter through

cotton-wool, as follows:—A fourfold layer of cotton-wool (of the kind used for bandaging) is laid across a funnel in such fashion that the wool projects over the edge. The funnel thus prepared is placed in the steamer for an hour and the hot agar poured on to it. Another method is to omit filtering, and to allow the fluid agar to stand in the steamer after turning out the flame, in which case the turbidity mostly deposits, and the clear supernatant fluid is drained off. Narrow and long cylinders are well adapted for this purpose; for one can allow the agar to solidify therein, and then push it out and cut away the opaque lower portion.

6. As for broth.

Repeated boiling does not injure the capacity of agar for solidifying. Agar melts between 90–100° C., and remains fluid down to 40° C., but if the temperature falls below this point it solidifies very quickly—almost suddenly. When agar solidifies it expresses a little fluid—so-called condensation fluid.

[The following method of preparing ordinary nutrient agar by softening it first with dilute acetic acid has been in use in Dr. Klein's laboratory for many years past, and is an improvement on the method described above.

Composition of the Medium:—Agar fibre 20 grams, Peptone (Witte) 10 grams, Sodium Chloride 5 grams, Meat Extract to 1 litre. *Directions:*—Place the agar in a clean flask of 3 litres capacity, add 500 c.c. of tap water, and $2\frac{1}{2}$ c.c. glacial acetic acid. Allow the agar to soak for 15 minutes, then drain off the acidulated water carefully by holding the hand over the mouth of the inverted flask. Wash the agar thoroughly

with four lots of water, draining carefully between each, and after the last washing allow the flask to stand inverted to drain for 10 minutes.

Now add 500 c.c. of the meat extract, peptone, and salt, to the washed agar in the flask; and place in the autoclave at 115° C. for 30 minutes, or in the steamer for 90 minutes; then add remainder of the meat extract, &c. Make alkaline with Liq. potassi (till blue litmus-paper is rendered slightly more blue), cool to 60° C., add the beaten white of an egg, heat in the autoclave for 45 minutes, or in the steamer for 90 minutes. Filter through a moistened Chardin's filter-paper (32 cm.) placing the funnel in a warm water jacket, put in tubes and steam the latter for 30 minutes on two successive days.]

Modifications in the preparation of Broth, Gelatine, and Agar.

1. The meat extract may be diluted up to about four times its bulk without interfering with its nutritive capacity. Meat extract may also be made from flesh of animals other than the ox or horse, and may be prepared from placenta, testicles of bulls (very cheap), &c.

2. Instead of meat extract prepared in the manner described above, Liebig's extract of meat (Lemco) may be employed in 1–2 % solution, in which case the addition of salt is unnecessary. The medium thus made from Lemco, however, is not so valuable as that made from meat in the manner described. For *water examinations* the following gelatine preparation has been officially recommended:—Dissolve Lemco 2,

peptone (W) 2, NaCl 1, in water 200. Heat for half an hour in steamer, and, after the fluid has cooled and precipitated, filter. Add to 900 parts of this fluid 100 parts of gelatine, and, after the gelatine has soaked and softened, place in the steamer for, at the outside, half an hour. To the warm solution add 30 parts of a 4% NaOH solution, then add the same drop by drop until blue litmus-paper is no longer reddened by the medium. Heat for quarter of an hour in the steamer, test the reaction, and correct if necessary. Add $1\frac{1}{2}$ parts of crystallized undecomposed soda, steam for $\frac{1}{2}$–$\frac{3}{4}$ hour, and filter. Fill into sterile tubes, 10 c.c. being poured into each, and sterilize these tubes by steaming them once for 15–20 minutes.

3. Maggi's granular bouillon has also been introduced as a source of nutrient media by Merck of Darmstadt under the names Ragitbouillon and Ragit-agar powder. From these useful media can be prepared by simply boiling and adding definite quantities of soda solution; special media, e.g. for cholera and typhoid organisms, can also be prepared. [Messrs. Baird & Tatlock and Messrs. Burroughs & Wellcome also supply various media in powder and tablet form which need the addition of water only and boiling.]

4. Neutralization with litmus as indicator is difficult because it is not easy to say when the blue paper no longer becomes red. More exact but more troublesome is neutralization with *phenolphthalein as indicator*, which may be effected as follows:—5 c.c. of the medium is placed in a flask, diluted with 45 c.c. of freshly-prepared distilled water, and boiled for three minutes over

a flame. Add thereto 1 c.c. of phenolphthalein solution (0·5 grm. phenolphthalein in 100 c.c. of 50 % alcohol solution) and titrate with $\frac{N}{20}$ NaOH or HCl until the fluid develops a brilliant red colour. Now add to the rest of the medium according to the result of this titration experiment normal NaOH or normal HCl until the reaction is neutral. Thereupon titrate again a sample of the medium (5 c.c.) as above and correct the reaction of the rest if necessary. Heat to boiling and test again. If the medium is now neutral or slightly alkaline to phenolphthalein it is strongly alkaline to litmus, since the peptone and diphosphate present in the medium are neutral or alkaline to litmus but acid or neutral to phenolphthalein. As media are most suitable for bacterial growth when neutral or slightly alkaline to litmus, media neutral to phenolphthalein must receive an addition of acid. Add 1·5% (up to 2·5%, note how much) normal HCl, boil, filter, and sterilize.

5. The addition of 2–8 % of glycerin puriss before sterilization increases for some purposes the value of a medium. Glycerin agar is the usual form of glycerinated medium employed.

The process of filling tubes with media and sterilizing them.

New test-tubes (those in general use are 160 mm. long by 16 mm. in diameter) should be boiled before use in 1–2% HCl, for the reason that when heated they are apt to give off alkali, which renders the medium turbid and unfit for use. It is advisable to purchase tubes of Jena

glass (Schott), which give off but little alkali. Old test-tubes are boiled in water or placed in the steamer. The tubes are cleaned by careful brushing, and are then allowed to dry, after which each is provided with a plug of wool about 3 cm. long, which is gently rolled in and allowed to protrude a little over the upper margin. They are then sterilized in the hot-air sterilizer as directed on p. 10. This sterilization, however, may be omitted in case of tubes that will be subsequently sterilized after being filled with nutrient media.

When pouring medium into tubes, the lower end of a funnel should be provided with a piece of india-rubber tubing fitted to a small glass tube. By means of this appliance (which is sterilized in the steamer with the medium, and provided with a clip at the rubber portion) one can readily fill the tubes without smearing their upper portion and thus causing the wool plug to stick to the glass. Each tube should contain about 5 c.c. of medium.

For filling tubes with definite quantities of medium either a specially constructed filling funnel (Treskow's) is used, or a graduated burette is filled with a tube junction and a ⅄ or ⊥-shaped nozzle, through one limb of which the fluid medium is allowed to flow up to a given mark. The flow is then shut off; and from the other limb, which is provided with a rubber tube and pressure clip, the required quantity is filled into the tube. Before sterilizing tubes filled accurately in this way, one should, as a precaution, mark the upper limit of their contents with a grease pencil and observe whether the quantity decreases during sterilization.

After being filled, tubes are sterilized in the steamer as a rule on three successive days for a quarter of an hour, but if the tubes and filling arrangement are sterilized, one steaming for a quarter of an hour suffices. After steaming, the tubes of agar and gelatine are either allowed to remain in an upright position, or they are sloped on a support (glass rod, &c.). When tubes are being sloped, care must be taken that the medium does not touch the plug of wool, or the latter will stick.

Peptone-Water.

As a substitute for broth, one can often employ a solution of peptone (Witte-Rostock) 1–2 %, and salt ½–1 % in either distilled water or ordinary water. For certain indol reactions (p. 50) one adds also 0·01% KNO_3 and 0·02 % crystallized soda. Sterilize in the manner directed for meat extract. This medium is well suited for cholera and similar vibrios, fairly good for typhoid and coli bacilli, but bad for diphtheria. For water investigations of certain kinds it is advisable to keep ready concentrated peptone solutions in sterile flasks (peptone 10, salt 5·0, grape sugar 10·0, water 100 for the detection of B. coli, and also peptone 10, salt 10, KNO_3 0·1, crystallized soda 0·2, water 100 for enrichment of cholera vibrios).

Blood Serum Media.

Blood gushing from a punctured wound in the neck which is made in killing an animal (bullock, sheep, horse) is collected in large glass cylinders

which have been rinsed out with sublimate, alcohol, and ether, or dry sterilized; and these vessels are allowed to stand for twenty-four hours in a cool place. With a sterile pipette the separated clear or slightly blood-stained serum is then removed and filled into sterile test-tubes. One can promote separation of the serum by loosening the blood clot with a sterile glass rod from the side of the vessel a few hours after the blood has been collected. The serum may be converted into a transparent solid medium by heating it for a variable time to about 70° C., the tubes being sloped in the special apparatus used for this purpose.

The serum thus collected contains almost always very resistant germs. In order to obtain serum free of bacteria one may employ fractional sterilization (p. 11, 4 (*b*)), but this process is not quite reliable. Another method is to place the tubes, after sterilization, in the incubator for twenty-four hours, and to eliminate those in which growth has taken place (frequently 50 % or more). An alternative method is to fill the serum into medicine bottles, to add plenty of chloroform, and close the bottle with a rubber stopper; then the serum is—but only after several months—certainly germ free. This method has the disadvantage that supplies of the serum must be kept in stock. Attempts to free serum of germs by filtration are tedious and time-wasting. The most successful way of obtaining sterile serum is to draw it off with absolutely aseptic precautions. Thus one ties a sterile cannula with aseptic precautions into the carotid of a sheep or calf in the laboratory, allows the blood to flow through a sterile india-

rubber tube into a sterile flask, and the serum is then dealt with in the way described. If fluid blood-serum is needed, it is best obtained in this fashion. Small quantities of blood can be obtained from large animals by carefully disinfecting the skin and passing a sterile cannula or exploring needle through it into the jugular vein.

For most purposes one can sacrifice the transparency of the solidified serum (which is on the whole somewhat feeble) and sterilize it after solidification in sloped tubes by heating to 95–98° C. for three successive days for half an hour in the serum inspissator; or one can heat it in the steamer to 100° C. straight away, but in that case the surface of the medium often becomes uneven from the formation of bubbles. However prepared, as a precaution one keeps the tubes before use for twenty-four hours at 37° C. and eliminates those which show contamination (turbidity of condensation fluid, &c.).

Instead of tubes, one may allow the serum to solidify in Petri dishes (p. 32), and sterilize as above, but in this case the surface of the medium dries up quickly, and extraneous organisms tend to find access to it.

Ascites, ovarian cyst, or hydrocele fluid obtained by aseptic puncture can be used in place of serum; the reaction, however, is sometimes very alkaline, and should always be tested.

Blood Serum with Broth (Loeffler's Serum).

The nutrient property of the blood serum is increased by adding to 3 or 4 parts of serum

one part of slightly alkaline broth containing peptone 1 %, salt 0·5 %, and glucose 1 %. This addition is made before the tubes are filled. The capacity of the serum for solidifying is not reduced by the addition of the broth, but rather higher temperatures (90–95° C.) must be employed for obtaining good solidification.

Human Blood Serum.

See under Gonococci (p. 161).

Serum-Agar.

Sterile fluid serum warmed to between 40 and 50° C. is mixed with agar (2-3 %) which has been melted and then cooled down to this temperature. The serum is mixed with the agar either in equal parts, or in the proportion of 1 of serum to 2 of agar. The medium, which solidifies on cooling, is either inoculated while still fluid and then poured into plates, or else it is first allowed to set in plates and in sloped tubes, and surface cultures are then made. Ascites or hydrocele agar is prepared in a similar manner (see also p. 162).

Eggs.

The shell of the egg is carefully washed and brushed in soap and water; it is then washed in 5 % sublimate and in sterile water, and dried with sterile wool. For the purpose of inoculation a small opening is made in the apex of the egg, and when the inoculation has been made through this, the opening is closed

with sealing-wax, or sterile paper and collodion. The medium is an unsatisfactory one, because it is frequently not sterile.

Solidified white of egg may be prepared either with or without the yolk. In either case the shell is sterilized. When preparing the white separately, the shells are broken and the white collected in the way that a cook does. When preparing white and yolk together, an opening is made at either pole of the egg, and the contents are then blown out into sterile test-tubes or into plates. The medium is solidified and sterilized in the serum inspissator in the same fashion as blood serum. For special egg media used for culture of tubercle bacilli see p. 97.

Potatoes.

1. *Halved Potatoes with Skin on (Koch).*

Good salad potatoes are cleansed with a brush under the water-tap. After the so-called eyes and decayed spots have been picked out with the knife, the potatoes are placed for half an hour in 1 per 1,000 sublimate solution. Then they are rinsed with water, heated for three-quarters of an hour in the steamer, grasped with sterilized hands, divided with a sterile knife in the zone of their greatest circumference, separated, and kept in a moist chamber (large double capsules with wet blotting-paper on the floor) in such a way that the individual halves of the potato do not touch. They are inoculated on the centre of the cut surface.

Many spores are attached to the outside of the potato, and some of these survive boiling, and tend to grow through the entire substance of the potato later.

Hence the two following processes in which the skin is removed are preferable.

2. *Potato Slices without the Skin (v. Esmarch).*

The potato cleansed as in No. 1 is peeled and divided into slices 1–2 cm. in length. The slices are placed in sterile plates, and sterilized in the steamer. It is safest to expose these pieces of potato to 110°–120° C. for one hour, but in this case they become browned and shrivelled. The alternative is to place them in the steamer at 100° C. for $\frac{1}{4}$–$\frac{1}{2}$ hour repeatedly.

3. *Potato wedges without the skin (Bolton Globig).*

With a corkborer one cuts out a cylinder from a cleansed potato, boring in the direction of its longest axis, after removal of the skin at the spot where the borer is to be inserted. The piece of potato removed inside the borer is then cut obliquely with a sterile knife, and two wedges thus obtained. These are placed in sterile test-tubes, the broad end of the wedge being at the bottom of the tube. In the lower end of the test-tube on which the wedge rests is placed either a plug of wool or a small piece of glass in order that the piece of potato shall stand free of the moisture which exudes from it when steamed. Sterilize as in 2.

4. *Potato Pulp.*

Boiled potatoes crushed into a pulp with the addition of either water or milk are filled into Erlenmeyer's flasks to a depth of about 1 cm., and sterilized in the steamer.

Potatoes have an acid reaction. For bacteria which

are susceptible to acid, therefore, potatoes form a better medium when they have been boiled in 3 % NaCl or 1% Na_2CO_3 solution afterwards poured away. For glycerinated potato see p. 90.

Bread.

Very dry white bread is powdered. Sufficient bread crumbs to cover its bottom are placed in an Erlenmeyer flask, and enough water added to produce a thick pulp. The bread medium is then sterilized either in the steamer or in the autoclave. It has an acid reaction, and is a good medium for moulds.

Milk.

Fresh milk, preferably with the cream removed, e.g. skim milk, giving an amphoteric reaction to litmus-paper, is poured into test-tubes and heated in the steamer on three successive days for $\frac{1}{2}$–1 hour. (At a temperature of 110° C. and over, the medium undergoes brown discoloration.) To prove its sterility, milk should be kept for at least three days at 37° C. before inoculation. For litmus-whey see p. 49.

Albumen-free Media according to Uschinsky and C. Fränkel.

NaCl 5·0, dipotassium or sodium phosphate 2·0, asparagin or aspartate of soda 4·0, ammonium lactate 6·0, dissolved in ordinary water 1,000 c.c. Neutralize and make slightly alkaline with NaOH. Sterilize as in the case of broth.

The Preservation of Media.

General Rules. Always label media with the date of manufacture, composition, and reaction.

Nutrient media frequently deteriorate on keeping, especially from evaporation, and from the penetration of moulds, which are apt to gain access despite a closely-fitting plug. Both these contingencies, however, can be avoided by flaming the margin and protruding plug of the tubes or flasks containing the medium directly after they have been steamed, and by then covering them with a closely-fitting cap of sterilized rubber, tinfoil, or metal. Another way of preserving media is to place the drawer containing them in a tin box (biscuit tin) with closely-fitting top, in which at the same time a large piece of blotting-paper is placed which has been saturated with oil of cloves.

Dried-up media may be renovated by adding a quantity of water equal to that lost by evaporation, and by then resterilizing them after thorough admixture.

Media beginning to get mouldy can also be saved by resterilization.

For special culture media see the following pages:—Drigalski and Conradi's Medium, p. 128; MacConkey's Bile-salt Media, p. 226; Nasgar, p. 160.

IV

Culture Methods

Plate Cultures.

THESE serve for the purpose of isolating from a mixture the different kinds of bacteria which may be present. The mixed bacteria are distributed in a solid medium which has been melted; and thus are held apart when the medium solidifies. Provided that the medium is favourable to them, such separated bacteria then develop into isolated colonies.

1. *Gelatine Plates.* Three tubes of gelatine are liquefied in a water bath at 30-35° C. One of these tubes is held in the left hand between the thumb and the palm which is turned upwards in an oblique position, the opening of the tube pointing to the right. The plug of wood is now removed from the tube and held between two of the finger-tips of the left hand in such a way that the part of the plug which is to be again introduced into the tube points downwards, and is not touched by the fingers. A tube should always be held in this way when it is being inoculated. With a sterilized platinum loop one then takes a trace of the bacteria-containing material and distributes it in the fluid gelatine contained in the upper portion of the tube. The loop is sterilized in the flame again and laid aside, and the plug of wool replaced in the tube. The inoculated

material is then distributed in the gelatine by gently moving and rotating the tube. Shaking should be avoided, for thereby bubbles are liable to be developed which become fixed in the gelatine and thus simulate colonies. The fluid gelatine should not be allowed to touch the wool plugs.

Now one grasps the tube in the same way as before, and also the second tube, and removes the plugs from both. With a sterile platinum loop three drops of tube 1 are then transferred to tube 2. The loop is resterilized, the plugs replaced, and tube 2 gently rotated to ensure distribution of the material. Tube 1 is now replaced in the water bath, and three loopfuls of tube 2 are transferred to tube 3.

If the material to be examined is very rich in bacteria, one sows less. One may transfer in this case only one or two loopfuls, or a fourth and fifth tube may be used. In this case tube 1 can contain water instead of nutrient media; such a tube being useless for plating when so heavily inoculated.

Now one pours out the gelatine from each tube into a dry sterile Petri dish or plate, not omitting to flame the top of the wool plug of the tube before removing it. The plate is opened by slightly raising the upper cover on one side, and quickly pouring in the medium through this opening. Petri dishes or plates have quite superseded the older form of flat glass plate, which had many disadvantages.

If one has no plates, then recourse may be had to roll-tubes. An india-rubber cap is in that case placed on the end of the tube, and the latter is held in a horizontal position under the water-tap, and rapidly

rotated so that the gelatine sets evenly all round the tube. The plug should not be touched by the gelatine, and to this end it is advisable in the case of roll-tubes to have only a little gelatine in the tube (5 c.c. at the outside), and to allow the gelatine to cool nearly to the point of solidification before rotating it. In summer the tube may be rolled in a capsule of iced water, in which case one must avoid touching it with the warm hand when it has set. Roll-tubes are unsuitable in the case of bacteria which rapidly liquefy gelatine.

Always mark all cultures at once clearly and carefully with a grease pencil, or better with a label, not omitting to record the date.

2. *Agar plates* are made in just the same way as gelatine plates, the agar being melted and then cooled to 40° C. Work quickly because the agar sets rapidly. It is well to warm the plates slightly before pouring in the medium. Roll-tubes of agar turn out badly. Agar plates are inverted before they are placed in the incubator because otherwise the condensation fluid which is expressed from the agar is apt to make all the colonies on the surface of this medium confluent. Condensation water can be absorbed by placing in the cover of the plate by means of sterile forceps a sterilized circular filter-paper of appropriate size.

For preparation of serum-agar plates see p. 25.

Advantages of gelatine. Colonies mostly grow in characteristic forms.

Disadvantages. It remains firm only to 30° C. at the outside; and made in the usual way it only remains firm up to 22–24° C., especially in plates. It is there-

fore not solid at the body temperature. The rapid liquefaction (peptonization) of gelatine produced by certain bacteria also interferes with the value of plates of this medium for isolating bacteria.

Advantages of agar. It can be used as a solid medium for the culture of bacteria at the body temperature. It is not liquefied, so that agar plates keep longer than gelatine.

Disadvantage of agar. The bacterial colonies are not as a rule characteristic.

An Economical Method of diluting Culture-material.

If one wishes to obtain dilutions with small consumption of gelatine or agar, one places a number of drops of broth or gelatine on a sterile plate and inoculates the first drop with the material to be examined, from this the second drop, from that the third, and so on. From given drops, one or more gelatine or agar tubes can then be inoculated and poured out as plate cultures.

The Examination of Plates.

Colonies developing in plate cultures are examined with low objective, narrow diaphragm, and concave mirror. In the case of Petri dishes one raises the cover; or, if one does not wish to subculture, the plate may be examined microscopically in inverted position.

Isolated colonies may be subcultured by the process known as *Fishing.* The selected colony is placed in

the field of a low-power microscope, and with a fine sterile platinum needle, the end of which may with advantage be bent, one touches lightly this colony, controlling the process by looking through the microscope. After the colony has been touched, the needle is removed as carefully as when brought to the colony. In order to avoid touching any other part of the plate than the selected colony, the little finger of the hand that guides the needle may be rested on some solid support. The material removed from the colony by the needle is used either for preparations or for pure cultures. If the colonies be not crowded, the selected colonies may be subcultured by touching them with the needle without the aid of the microscope.

A more comprehensive investigation of the growth on a plate may be effected by means of *Impression-preparations*, which are made in the following way. A cover-glass is sterilized in the flame, allowed to cool, pressed firmly down on the plate and then raised with the forceps. The preparation thus obtained is stained according to the directions given later (p. 58). Impression-preparations afford general information concerning the kinds of bacteria present, and are especially useful for the study of the smallest colonies. Subcultures must not be made from a part of the plate where an impression-preparation has been made.

For *Enumeration of Micro-organisms* see pp. 208, 217.

The Preparation and Subculture of Pure Cultures.

Preparation from plate cultures. One fishes some

material from an isolated colony and inoculates therewith tubes of sterile medium. In fluid media one cleanses the needle by moving it from side to side; in the case of solid media one either moves the needle over the whole of the surface, or one draws it along the middle in a straight line—*Streak Culture.* Avoid scratching the surface of the medium. *Stab Cultures* are made by puncturing transparent medium which has solidified in a tube kept in an upright position. If the growth on the stab is to be examined under the low power of the microscope, the stab is made near the wall of the tube; otherwise it is made in the centre.

Subcultures are made by transferring small quantities of the growth with the sterilized platinum needle or loop into fresh tubes of culture media. When making subcultures, the tubes are held in the manner previously described, gelatine tubes being held with the open end facing downwards.

For the purpose of keeping bacteria in culture, transference to fresh media, or, as the process is termed, subculture, is as a rule sufficient every 4–6 weeks; but some of the pathogenic bacteria (influenza, gonococcus, pneumococcus, and meningococcus) require more frequent transference. If the culture has dried up one adds a little sterile broth, incubates 24 hours, and then subcultures. Bacteria, however, will retain their vitality for months when one saturates small silk threads (No. 4 size) with cultures of them, or better, with blood or other albuminous fluid in which they are contained. These threads are dried in sterile capsules in a desiccator over $CaCl_2$, and are

then kept in small test-tubes which are placed inside larger tubes having $CaCl_2$ at the foot and closed with a plug of wool and rubber cap.

On the employment of Surface Plate cultures for the cultivation of fractions of the material under examination.

Instead of distributing the bacteria-containing material in melted nutrient media, and then pouring this out into plates and allowing it to set, thus fixing the individual bacteria apart, one can effect the isolation of such bacteria by spreading out the material on the surface of media which has already set either in plates or in sloped tubes.

This is effected by using a loop, or a sterile plug of wool, or a heated platinum wire, or a piece of capillary pipette bent in the flame, and by this means distributing the material over the whole of the surface of the medium. Provided that the original material is not too rich in bacteria, in the case of plates, where the surface of the medium exposed is comparatively large, it is usually easy to obtain isolated colonies by sowing a single plate. In sowing such a plate a drop is placed on the medium with a loop, and this drop is then thoroughly distributed with a wire bent parallel to the surface of the medium : glass rods bent to a right angle are also suitable for distributing material over a plate. In the case of sloped tubes, however, it is as a rule necessary to inoculate a series in succession, using the condensation fluid as diluent. Thus in inoculating a series of serum

tubes one would add a loopful of the material to the condensation fluid of the first tube, and then distribute a loopful of this condensation fluid over the whole surface of the serum in this tube; then with the same needle, without recharging it, a second and third serum tube are dealt with in the same way in quick succession. The third tube will generally be found to yield a crop of well-separated colonies from which pure cultures can be made. This is the sole method of isolating bacteria in pure culture from media which cannot readily be converted from a solid to a fluid state and vice versa, such as blood serum, and potatoes.

Single Cell Cultures (Burri).

By the plate and streak methods one is not certain that each individual colony arises from one organism only and not from several. Reliable cultivation from a single organism has been rendered possible by the Indian-ink method of Burri (*Das Tuscheverfahren*. Jena: G. Fischer, 1909). Pelican ink no. 541 (Grübler) 1 and distilled water 9 c.c. are placed in a test-tube, sterilized in the autoclave, allowed to stand for two weeks and the upper portions of the suspension are removed for use. With a large platinum loop dipped after heating in sterile water one places four separate drops of the Indian-ink suspension on a grease free slide. One now quickly adds a portion of the bacterial emulsion to drop 1, and with a small loop transfers a portion to drop 2, some of this to drop 3, and so on. One now takes a fine mapping pen, heats it in the flame (but not to redness) and when cool makes

several rows of small dots on a well solidified gelatine plate. After half a minute this is covered with a sterile cover and examined with a high-power dry lens. The bacteria appear bright against the dark particles of Indian-ink. One marks on the under side of the plate those dots which contain one organism only, allows the bacteria to develop into colonies, and subcultures from those colonies which arise from a single cell.

If the cultivation is to take place on some other medium than gelatine, the dots are first made on a gelatine plate as above and each dot is then covered with a sterilized piece of coverslip. One raises the fragment under which the microscope reveals but one organism and transfers it to the desired culture medium – the Indian-ink and bacterium adhere to the coverslip.

Drops of Indian-ink containing bacteria are also well adapted to the investigation of fine bacteria, &c, which appear brightly illuminated between the particles.

For single cell cultures of yeasts see p. 167.

The Animal Body as a means of obtaining Pure Cultures.

Some pathogenic micro-organisms can be isolated from mixtures by means of the animal body. For this purpose it is necessary that no other pathogenic germs which can penetrate to the inner organs of the animal should be present in the mixture. In a successful case, after inoculation, only one kind of micro-organism multiplies in the tissues of the animal, and after

the animal has succumbed, this organism is recovered from its inner organs in pure culture. The method is applicable for the isolation of anthrax, mouse septicaemia, tuberculosis, plague, and the pneumococcus. In making cultures from the animal body, it is generally better not to attempt to make pure cultures direct, but first to prepare plates. If one does attempt to make pure cultures direct, stab cultures should not be made, but slope cultures, in which contaminations are more readily perceived if present. Never cultivate in fluid medium, because here impurities are not so easily recognized.

Hanging Drop Cultures.

These are made in broth, gelatine, &c., for the purpose of directly observing the growth of bacteria under the microscope. For method of preparation see p. 1.

The Cultivation of Anaerobes.

To media employed for the purpose of cultivating anaerobic bacteria one adds as a matter of routine 1–2% grape sugar, and, as well, 0·3–0·5% sodium formate, or 0·1% sulphindigotate of soda. The latter stains the medium blue, but owing to a process of reduction, the colour is usually discharged by the growth of bacteria (p. 50).

A good proportion of anaerobic bacteria are spore-formers. Material from which one wishes to isolate them therefore is first examined microscopically, and if spore-bearing anaerobes are seen (the form of which

is generally obvious) then by heating the material to between 55 and 60° C. for half an hour one can without injury to the obligatory anaerobes kill the numerous vegetative forms of facultative anaerobes mingled with them which one does not wish to cultivate. In this way the obligatory anaerobes can eventually be obtained in pure culture. But cultures should also always be made from the material unheated.

Of the numerous methods for culture of anaerobes the following moderately successful ones may be described:—

1. Methods in which air is not excluded.

Several anaerobes can be cultivated without exclusion of air in broth tubes containing a deep layer provided one thickly sows the deepest layers of the freshly boiled broth without shaking and adds reducing substances to it; as such one may use pieces of liver, spleen, kidney, brain, boiled egg, potato, or tissue emulsion, or even platinum sponge. The substance added should not be too small in quantity (about 1 gr. to 10 c.c. broth): it is sterilized with the broth. Inoculation should take place immediately after cooling, and the tubes should be protected from shaking as much as possible.

2. Methods in which the Oxygen is removed mechanically.

(*a*) *Cultivation in the depth of media.* Test-tubes are filled one-quarter full with gelatine or agar, thoroughly boiled, quickly cooled in water, and, without shaking, inoculated. This method is suitable for the

isolation of anaerobes if the material containing them is well distributed throughout the medium when still fluid, and is well diluted, e.g. by inoculating 2–3 tubes in direct succession without allowing air to get in whilst shaking. After the medium has set, one pours in carefully (after flaming the mouth of the tube) the melted contents, which must not be above 40° C., of a second and third tube. In the case of gelatine, it is best to pour in, not gelatine, but agar. For the purpose of examining and isolating the colonies that develop, the glass is broken and the medium divided into pieces at the desired point with a sterile knife, or the isolated colony may be approached from above without breaking the glass, by means of a long needle or capillary pipette.

For the purpose of keeping up a pure culture, stab cultures are employed (stab till the deepest layers are reached); the tubes being filled about three-quarters full of the medium and freshly boiled.

Air having been expelled from the medium by boiling, sterile oil may be poured on the surface in order to prevent further access of it. Many anaerobes will grow in freshly boiled fluid medium under a layer 3 cm. high of oil, liquid paraffin, or vaseline, but the method is not a clean one, for the grease interferes with subculture. For further modifications see Ghon and Sachs, *Centr. f. Bakt.* I, Or. 32, p. 403.

Anaerobes can also thrive in the closed limb of a fermentation-tube. The same holds for other fermentation vessels (see p. 47).

(*b*) *The air is removed by an air-pump* (Grüber). After inoculation, the tubes are placed in a water

bath at 30–35° C. in case of gelatine, and at 42° C. in case of agar. A paraffined rubber stopper, through which passes a glass tube connected to the air-pump and ending flush with the under surface of the stopper, is then inserted into each tube, and the air exhausted from it. As the air becomes rarefied, the medium begins to boil. After about a quarter of an hour the tube is exhausted sufficiently, and it is then closed by sealing it in the flame at a point where it has previously been drawn out. In case of gelatine, roll cultures can now be made if desired. (For this method combined with No. 2, see Emmerling, *Hyg. Rdsch.* 1904, p. 452.)

3. Method in which the Oxygen is absorbed (Buchner).

The culture tubes or Petri dishes are thickly sown and then placed in a vessel which can be closed hermetically, e. g. either a wide test-tube with greased rubber stopper, or an exsiccator. The Petri dishes remain open, and are placed one above the other, being separated by glass plates. The vessel should contain pyrogallic acid, and after inserting the cultures, caustic potash is added to this, and the vessel immediately closed. The alkaline pyrogallic solution absorbs the oxygen. For absorption of the oxygen from 100 c.c. of air, one allows 1 gram of pyrogallic acid (dissolved in 2-3 c.c. of water) and 10 c.c. of the following preparation of caustic potash:—Liquor kal. caust. (15 % KOH) 1·0, aq. 10·0; or, according to Hammerl, pyrogallic acid 2 grams dissolved in 1·5 c.c. of 15 % KHO. Complete absorption of the oxygen

requires twenty-four hours at blood heat, but if one adds the potash warm, this is effected quicker. For double capsules suitable for O absorption, see Schüler, *Centr. f. Bakt.* Abt. I, Or. 37, p. 298; and Dreuw, ibid. 36, p. 748. For absorption by means of phosphorus, see Sellards, ibid. 37, p. 632. For single dishes the method of Lentz is well adapted (*Centr. f. Bakt.* I, Or. 53, p. 358). On a glass plate one lays a thick piece of blotting-paper soaked in pyrogallol (Pyrogallolfilz of Lautenschläger, Berlin), which one surrounds with a thick ring of plasticine. One now adds 15 c.c. of 1% aqueous KOH and immediately presses down the inoculated plate over the plasticine ring and seals it well round with plasticine. The plate can be examined microscopically without breaking the seal.

4. Methods in which the Oxygen is replaced by Hydrogen.

(*a*) *By passing hydrogen through* (Fränkel, Hüppe). The sterile flask or tube, which is provided with medium and inoculated, contains in its neck a rubber stopper which is perforated by two glass tubes. One of these, which is bent to a right angle above the stopper, passes down into the medium; while the other, which is also bent, is cut off flush with the under surface of the stopper. In structure the apparatus thus resembles a wash-bottle. Through the first tube is passed hydrogen generated from a Kipp's apparatus, and purified by passage through potassium iodide solution and through a solution of pyrogallic acid and caustic potash. Hydrogen is passed through the

culture until the gas that exudes at the outlet—which is drawn to a fine point—burns with an even flame and without noise. Thereupon, without stopping the flow of hydrogen, this point is fused in the flame. The rubber stopper may be covered with melted paraffin to ensure its being air-tight. After the air has thus been replaced by hydrogen, if the medium in the flask is gelatine it may be distributed over the walls in the manner of a roll-tube. The worker is warned against an explosion which may occur if the gas at the outlet is lighted too soon. To avoid this, it is best to sample first the exuding gas by holding an inverted small test-tube over the outlet and igniting its contents in the flame. If the gas in the test-tube burns up quietly with very little hissing, it is hydrogen, and one may then ignite the gas coming out at the outlet.

Modification of the method for plate cultures (Blücher). A sufficiently large glass capsule is filled to a depth of about 1 cm. with pyrogallic acid solution. The inoculated plates of agar or gelatine are placed, with covers off, on a wirework support, which stands in the middle of the capsule on a raised platform with its lower border at least 1 cm. above the fluid. Over the plates an inverted funnel is placed in such a way that its rim dips into paraffin wax all round ; and in order to ensure this the funnel is loaded with a weight. Through the tube of the funnel hydrogen is then passed and the air expelled under its rim. After hydrogen has been passed through for some time, the rubber tube by which it is conveyed to the stem of the funnel is clamped by means of a pressure clip, divided, and the divided

portion of it down to the clip filled with melted paraffin. Lastly, by means of a pipette, liquid KOH is allowed to flow on to the pyrogallic, and melted paraffin is poured into the space between the capsule and edge of the funnel. Botkin (*Zschr. f. Hyg.*, Bd. 9) and Novy (*Centr. f. Bakt.*, Bd. 16) have designed large apparatus on a similar principle for holding a number of anaerobic cultures.

(*b*) *With addition of hydrogen* (Fuchs). The inoculated tube of medium is held in an inverted position in a clamp. In the case of serum and agar, therefore, the condensation fluid must first be removed. Hydrogen is now allowed to flow into the tube for several minutes by inserting the nozzle of a glass tube connected to the hydrogen generator into the tube and carrying it up to the top. The nozzle is then gradually withdrawn, and when it has reached the mouth of the tube, this is at once closed by a sterile rubber stopper and quickly immersed in a bath of melted paraffin. After removal from the paraffin, the tube is kept standing on its stopper end, i.e. inverted. If the method is used for agar it is best to employ old and rather concentrated agar, containing 0·5 % of gum arabic, so that it does not slip down upon the stopper and buckle up when the tube is inverted. The method is applicable for isolation of anaerobes by surface culture.

Methods for the Study of Special Biological Characters of Bacteria.

1. Examination of the Oxygen requirement.

Cultures are made under anaerobic conditions, in

which case obligatory aerobes fail to grow. As a control it is necessary to make at the same time cultures on the same medium to which air has access. Other simple tests of this matter can be made by determining whether growth occurs in the closed limb of a fermentation-tube, or in the depth of agar or gelatine.

2. Fermentation (Gas production).

Media containing from 0·25–0·5 % of glucose or other sugar are used. If one wishes to examine the action of bacteria on various sugars, such media should be proved to be themselves sugar free before adding sugar. For this purpose, therefore, one prepares broth from old decomposed meat, and then tests a sample of the medium with B. coli in a fermentation-tube. Should fermentable sugar be present, the broth is inoculated with B. coli, incubated for twelve hours at 37° C., boiled and filtered (in some cases through porcelain). It is then tested anew in a fermentation-tube with coli, and if gas development occurs the process is repeated. Finally, the particular sugar is added. The neutralization should be effected with caustic soda or sodium biphosphate. Carbonate of soda should not be used, for in that case CO_2 may possibly be given off in bubbles, through decomposition of the carbonate by the acid which is formed during the growth of bacteria, and such gas might be confounded with gas yielded by fermentation of the sugar.

Solid media containing sugar become fissured by gas bubbles formed by the action of fermenting bacteria, which may be sown either by distributing them in the medium when melted, or by stab culture.

In special fermentation tests one uses V-shaped glass tubes, the longer limb of which is closed—so-called fermentation-tubes. The long limb, which must be completely filled with the fermentable liquid, collects the gas; the short limb, which is provided with a wool plug, should contain only a small quantity of fluid, and should also possess a spherical enlargement above the level of this fluid, since it must receive the fluid driven out of the closed limb by fermentation. A further method is to use broth tubes containing a rubber stopper which presses on the broth, and is pierced by a glass tube closed with a wool plug, and either from 30–40 cm. long, or shorter, wider, and with a spherical end. The gas that develops under the stopper forces the broth up the tube. If there is no air space between the stopper and broth, this method may also be used for anaerobic cultures.

3. Alkali or Acid Formation.

Qualitative. The simplest way is to compare the reaction given to litmus-paper by the inoculated tube and by an uninoculated tube of the same medium. For quantitative titration see above, p. 20, 4, $\frac{N}{10}$ or $\frac{N}{100}$ acid or alkali is used, and litmus or phenolphthalein as indicator.

For demonstrating change of reaction brought about by the growth of bacteria, one may add to the medium a solution of litmus or azolitmin (1 : 100 aq.), and arrange the reaction in such fashion that a drop of $\frac{N}{10}$ acid or alkali produces a distinct red or blue

colour. The medium is then sterilized and re-tested, and if a change of reaction has taken place, it must be corrected, and the medium again sterilized and then inoculated. Litmus-tinted media often become decolorized, especially in the depth, by reduction brought about by the growth of bacteria. In such a case the observer should shake the tube and see whether the colour returns.

Change of reaction may also be observed by using Petruschky's litmus-whey, which is prepared as follows:—Milk is warmed to 40–50° C. with an equal part of water, and just enough (not too much) diluted HCl is then added to precipitate all the casein. The precipitate is filtered off, and the medium carefully neutralized with soda solution. Heat for 1–2 hours in the steamer, filter till clear, and if necessary correct the reaction. The tint of the whey should vary from the transparency of water to greyish-yellow. Sterile litmus solution should be added until a violet hue is produced. The medium should then be sterilized.

For particulars of further colour media applicable for demonstrating changes of reaction produced by bacteria, see under typhoid and dysentery bacilli (pp. 115, 137), pyogenic cocci (pp. 153–6), water (p. 222).

In the case of agar and gelatine, acid formation may be demonstrated by adding finely pulverized and sterilized chalk to the medium before inoculation. Clearing of the medium in the neighbourhood of a colony or culture indicates acid formation.

4. Reduction.

This is tested by cultivation in a medium to which

has been added some substance easily discoloured by reduction, e.g. sulphindigotate of soda (p. 40), litmus, neutral red, or methylene blue. Not more than 1–2 drops of a 1% solution of methylene blue should be added per cent. of the medium, or growth may be impeded. The colour is only discharged in those parts of the medium to which air has no access: accordingly anaerobic cultures are best for observation of reduction. Fluid media that have lost their colour from reduction regain it on being aerated by shaking.

5. The Formation of Sulphuretted Hydrogen.

About 3% of iron tartrate may be added to the medium. (This iron tartrate may be freshly prepared by throwing out $Fe_2(OH)_6$ from a solution of Fe_2Cl_6 by addition of KOH. The precipitate is washed, pressed in a cloth, dissolved in tartaric acid, and added to freshly prepared gelatine which is then sterilized in a steamer.) Another method of testing for H_2S is to suspend a piece of moistened lead paper under the plug of a culture tube; or the lower end of the plug may be wetted with boiled sugar of lead solution. A darkening of the medium, or of the paper, or of the plug, indicates the presence of H_2S.

6. Indol-formation.

To a pure culture of the micro-organism in broth or peptone water is added 1 c.c. of 0·01% sodium nitrite solution, and 1 c.c. of purest sulphuric acid (1 + 3 aq. dest.) or HCl. Red coloration within five minutes indicates that indol is present. This reaction is particularly sensitive when performed as a 'ring' test,

in which case the nitrite solution is poured last on to the surface of the liquids. Some bacteria, for instance, the cholera vibrio and similar organisms, form indol by reduction from traces of nitrate in the peptone. Their cultures therefore give red coloration on addition of sulphuric or hydrochloric acid alone without $NaNo_2$ —the so-called nitroso-indol reaction.

The following test for indol (Böhme, *Centr. f. Bakt.*, Bd. xl, 1905) is an improvement on the above, as cultures of B. coli communis in peptone water give a positive reaction with it within forty-eight hours at 37° C. Solution A. Paradimethylamidobenzaldehyde 8 grams; HCl pure 160 c.c.; alcohol abs. 760 c.c. Solution B. Potassium persulphate cold saturated water solution. To test a culture for indol, add 1 c.c. A, shake up, and add about 1 c.c. B. Leave for two or three minutes: a rose colour indicates presence of indol.

Morelli's test :— Strips of filter-paper are dipped in warm saturated aqueous oxalic acid solution, after cooling they are fixed in the culture tube by means of the plug so that they hang freely. If indol develops the strip becomes coloured red. This test is available for all media, but in gelatine indol-formation is slow and scanty.

7. Proteinochrome-formation.

Cultures in 5% peptone broth or in 3% peptone water are slightly acidulated with acetic acid, and then fresh saturated chlorine water is added drop by drop, or chlorine water is gently poured on the surface. The development of a red-violet colour, or in the case of chlorine water being poured on the top,

the development of a red-violet ring at the point of contact, indicates the formation of proteinochrome. (Erdmann and Winternitz, *M. m. W.* 1903, p. 982.)

8. Light-emission (phosphorescence).

It is advisable to cultivate on gelatine or agar made with fish broth (meat broth will do) with 1% peptone, 0·5% glycerine, and 3% NaCl or KCl and neutralized. Surface cultures must be made, as the free access of air is necessary. Observe the cultures in a darkened room for at least several minutes, as frequently the light only becomes perceptible by degrees. Some strains of phosphorescent bacteria shine only in quite young cultures, and then but for a short time.

9. Resistance to Heat and Desiccation.

Resistance to moist heat. Capillary tubes are filled from a well-developed culture of the micro-organism in some fluid medium. These capillary tubes are then sealed at one or both ends in the flame, and exposed for a given time to the required temperature either in a water-bath or in the steamer. The capillary tubes are next washed in sublimate, alcohol and ether, and transferred by means of sterile forceps to tubes containing suitable medium, and are broken up therein by means of a sterile glass rod. The culture tubes should be kept under observation for several days in order to determine the question of growth. Spores always withstand 60° C. for over half an hour, and frequently withstand 80° C. for ten minutes. If desired, whole broth cultures can be heated as described above. They must be submerged in the

water-bath in such manner that the upper margin of their contents is well below the surface of the water. Place a thermometer in a control tube for the purpose of detecting any fluctuation of the temperature. After the heating has been completed, cool quickly. Such heated cultures are with advantage incubated for several days at a suitable temperature before inoculating other tubes from them, for in this way any isolated individuals possibly surviving in the broth culture have the opportunity of multiplying, and their presence is readily detected on subculture into fresh broth.

Resistance to desiccation. Broth cultures, or finely and evenly separated growth from cultures on solid media, are distributed upon broken pieces of sterile cover-glass, which are then placed in a sterile capsule and allowed to dry. From time to time one of these infected pieces of cover-glass is thrown into nutrient solution and growth determined. The effect of dry heat can also be examined in the same way. Similarly, sterile silk threads infected with cultures and allowed to dry have been employed to test resistance to drying, dry heat, and steam. In the last case the threads may be enclosed in rolls of blotting-paper and exposed in the steaming apparatus to steam at a temperature of 100° C. for a definite time (for threads infected with anthrax spores see p. 89).

10. Resistance to Chemical Disinfection.

The antiseptic value of a disinfectant can be determined by making cultures upon a medium which contains a definite proportion of the disinfectant.

The bactericidal power of a disinfectant is most simply tested by exposing either small pieces of glass, or silk threads upon which the bacteria to be tested have been dried, to solutions of different strength, and allowing contact for various periods. Before the material thus treated is sown in nutrient solution, it should be washed in sterile water, or in a solution that binds the disinfectant but has in itself no disinfectant value; or it should be sown in a rich nutrient fluid and thoroughly shaken.

Rideal-Walker coefficient.

In order to obtain some numerical value for the disinfecting power of a given substance a method has been devised in which the disinfecting power of various concentrations of the solution is tested side by side with that of standard solutions of carbolic acid. The ratio of the dilutions of those solutions which are of equal power is expressed as a number—the Rideal-Walker or carbolic acid coefficient.

Solutions of carbolic acid 1 in 20 and 1 in 100, and solutions of the disinfectant in varying dilutions up to 1 in 2000 are taken and 5 c.c. of each are placed in a series of sterile tubes. A broth culture of the test organism—most commonly B. typhosus or B. coli—i also required. Of this culture a large loopful is added to each tube of carbolic or disinfectant solution and thoroughly mixed with it. At intervals of 2 or $2\frac{1}{2}$ minutes a loopful is taken from each tube and inoculated into a tube of sterile broth. In this way some six to ten tubes are inoculated from each dilution of the disinfectant. The tubes are now incubated for 24-48 hours and examined for growth. The results

are set down in tabular form and a comparison is made in the following manner. Suppose the 1 in 100 dilution of carbolic acid has sufficed to kill the organisms in twelve minutes but not in ten; the table is examined to find what dilution of the disinfectant has also sufficed to kill within the same time limits. Suppose a 1 in 1200 dilution has so acted, then the Rideal-Walker coefficient is $\frac{1200}{100}$ or 12 for the organism specified.

It is advisable to use a large loop in carrying out the test, otherwise a fallacious result may be obtained in cases where the number of organisms has been very greatly reduced, so that a small loopful may fail to contain any living organisms.

[Before drawing inferences as to the practical value of a disinfectant, its germicidal action in the presence of albumen should be determined.]

11. Pathogenicity.

For examination of this see p. 205.

12. Toxin-formation.

Toxin may be dissolved in the medium (soluble toxin), or it may be confined to the bodies of the bacteria (endotoxin). *Soluble toxin*:—To demonstrate the presence of this, cultures in fluid media are filtered free of bacterial bodies (see p. 11, 4 (*c*)) or the bacteria are centrifuged out, the fluid is then covered with toluol, or phenol added up to 0·5% and allowed to stand for several hours; the fluid is then tested on animals (see p. 205). *Endotoxin*:—Cultures on solid media are killed by chloroform or toluol vapour. This

may be effected by pouring a few drops of chloroform or toluol on to the under side of the plug of wool with which the tube is plugged, by covering the tube with a double rubber cap, and then placing it for one to several hours in the incubator at 37° C. (Take care that the other cultures in the incubator do not suffer.) The killed growth may then without any admixture of medium be inoculated into animals. A control culture should be made to prove that all the germs are killed. (For dose to be inoculated see p. 207.)

[Endotoxin can be liberated from the bodies of bacteria by promoting autolysis (e.g. by NaCl); by dissolving their substance in NaOH; or by grinding them up in a special apparatus (McFadyean and Rowland) at the temperature of liquid air which apparently renders them brittle.]

V

Staining Methods

The Making of Preparations.

1. Cover-glass, Smear, or Film Preparations.

The Film. On the centre of a carefully-cleaned cover-glass (for slide preparations see p. 60) a drop of water is deposited by a platinum loop, and to this drop is added by means of a loop or needle a trace of the material under examination. The drop is then spread out over the surface of the cover-glass to form a uniform thin film. This is only possible when the

cover-glass is absolutely free of grease. The cover-glass is then allowed to dry, and this may be accelerated by gently warming it over the flame, with the film side upwards.

Fluids that do not contain too many bacteria—blood, pus—are smeared directly over the cover-glass undiluted. In the case of organs, a small piece is seized with sterile forceps and lightly smeared on a cover-glass. Materials which distribute over the cover-glass with difficulty, such as some kinds of sputum, nodules of tumours, and certain cultures, can be squeezed between two cover-glasses which are freed from one another by holding them in a pair of Cornet's forceps. Take care to avoid smearing the fingers. (For impression-preparations see p. 35.)

Fixation. The cover-glass with the film side upwards is held between the finger and thumb (or if the edge has been smeared, in the forceps) and passed three times through the flame. The advantage of holding the preparation between finger and thumb is to ensure that it is exposed to the right temperature. After being fixed in this way the film is ready for staining. Fixation can also be effected by alcohol; but the process takes longer. In case of blood preparations, it is recommended that they should be fixed by being left in equal parts of alcohol and ether from 2–10 minutes or longer.

Fixation by means of osmic acid is of value in obtaining good preparations. In a shallow glass jar with a tightly-fitting lid is placed a small dish covered with wire net and containing 5 c.c. 1% osmic acid (10 drops of glacial acetic acid should be added for blood

films). The still moist cover-glass preparation is laid on the net film side downwards for ½–1 min. It is then washed with quite dilute permanganate solution, dried in the air and stained. The wire net should be heated to redness each time it is used. The osmic acid solution remains active for several weeks.

Should doubt arise as to which side of the cover-glass the film is on, a hint may be gained by breathing on it ; the moisture condenses unevenly on the film side, evenly on the other. Or the cover-glass may be scratched with a needle.

Staining. For purposes of staining it, one takes hold of the cover-glass with a Cornet's forceps—an instrument usually closed, but opening on pressure. With a pipette (which should not be allowed to touch the film) one then drops on to the cover-glass sufficient stain to cover the whole of its upper surface. As a rule, one allows the stain to act for five minutes in the cold, or for 10–60 seconds over the flame (stains, p. 68). Another method of staining is to allow the cover-glass to float film downwards on some stain in a watch-glass ; and in cases where prolonged staining is needed the watch-glass may be warmed on the wire gauze of a tripod.

Washing. After having been stained, the cover-glass preparation is washed in tap-water, dried with blotting-paper, mounted in water, and examined microscopically. If, on microscopical examination, parts of the preparation move about and vibrate, it is a sign either of under-fixation or of over-washing. Should the water between slide and cover-glass evaporate, it can be renewed by placing a drop of

water at the edge of the cover-glass. Should it be necessary to treat the preparation with other fluids after straining, one either drops these on to the film, or dips the cover-glass into a vessel filled with such fluid.

Mounting in balsam. If desirable, cover-glass preparations can be examined in balsam. In this case, after the preparation has been stained and washed, it is dried either by allowing it to drain on a piece of blotting-paper and then to dry in the air, or by pressing it between two pieces of blotting-paper and then lightly brushing it with a camel's-hair brush. In the last case care must be taken not to injure the film. Having dried the preparation, a drop of viscid Canada balsam (dissolved in xylol) is placed upon the film side of the cover-glass, and the latter placed upon a clean slide. The balsam ought not to run out at the edges of the cover-glass, but should it do so, the exudation may be wiped off after a few days by a piece of blotting-paper steeped in xylol. In the same way immersion oil may be removed from the surface of the cover-glass. When mounted in balsam, bacteria appear smaller than in water, and some peculiarities, e.g. capsules, disappear. It is a good rule therefore always to examine in water first.

Preparations mounted in balsam keep for an indefinite time. They should be preserved in the dark. The stains gradually fade, especially methylene blue. Always label preparations at once.

When it is wished to mount in balsam a cover-glass preparation that has been examined in water, one removes any immersion oil from its surface by means

of blotting-paper, runs water round the edge of the cover-glass, and floats it off the slide. The cover-glass is then dried and mounted in the fashion described above.

Slide, i.e. Object-glass Preparations.

These may be made in the same fashion as cover-glass preparations, and are recommended in cases where one has to examine a large quantity of material and has only a single occasion on which to do so. The film is fixed and stained in the same way as in the case of cover-glass preparations. The slide is then dried with blotting-paper, or in the air, immersion oil is dropped on to it, and the film is examined direct without using a cover-glass. Should it be wished to preserve the preparation, the most important part of the film is covered with a drop of balsam and a cover-glass dropped on to it.

An estimate of the size of bacteria is most simply made by comparing them with red blood corpuscles the diameter of which varies from 7–9 μ. In cases where red blood corpuscles are not present in the material, e. g. in preparations from cultures, a drop of blood from the finger may be mixed with it.

2. Sections.

Hardening. Small pieces of tissue—at the outside as big as a phalanx—are hardened for at least three days in absolute alcohol ; the latter being frequently changed. It is well to place a pad of blotting-paper on the bottom of the vessel, so that the pieces of tissue by resting upon it may be kept in the upper layers

of the alcohol, which are more free of water than the lower. Pieces of tissue can be preserved for years in this way. In order to fix them better, one can, if necessary, place them in formalin for 12–24 hours before putting them into alcohol. (For a quick method see p. 63.)

For cutting, a microtome is used, and one endeavours to obtain very thin sections. The pieces of tissue, after being hardened, are attached or imbedded; and for this purpose the following methods are recommended; of which 1 and 2 should be used only for firm and parenchymatous tissue, 3 and 4 more especially for cavernous tissue, or small pieces of tissue.

1. *Attachment by glycerine-gelatine.* 10 grams of gelatine are dissolved in glycerine 40 + aq. 20 by heat. A drop of this is placed on a bit of cork, the piece of tissue is pressed upon it, and, after two minutes, the whole is placed in absolute alcohol. In the course of a few hours the glycerine-gelatine solidifies, and the material can then be cut. The sections are placed in a capsule containing 50% alcohol, and the knife also should be moistened with the latter.

2. *Imbedding in celloidin.* After being hardened in alcohol the pieces of tissue are placed for 1–8 days in a thin solution of celloidin made up by dissolving the latter in equal parts of alcohol and ether. They are next placed for the same length of time in a more concentrated solution of celloidin, and are then, by means of a spatula, transferred with the attached celloidin to pieces of cork or wood. The imbedded material must not be pressed upon when moving it.

When, after some time, the celloidin has dried somewhat in the air, the pieces are placed in 50–60% alcohol (not absolute) in order to allow the celloidin to solidify. Twenty-four hours later sections may be cut. The sections are kept in a capsule filled with 50% alcohol, and the knife is moistened with the same.

3. *Imbedding in paraffin.* The hardened pieces of tissue are placed for from several hours to days (until they become transparent) in xylol, then for the same time in paraffin dissolved in xylol, then in melted paraffin at a temperature of about 50° C. (Paraffin with the required melting-point can be obtained by mixing different samples.) The pieces of tissue are kept in the paraffin oven at 50° C. for 1–2 hours. After being soaked in this way in the paraffin, the piece of tissue is taken out with a spatula and placed on a clean glass plate inside a small mould made of glass, metal, or papier-maché, and melted paraffin is then poured round and over it in such a way that the piece of tissue lies in the middle of the mould. The glass plate may be dipped into water to hasten solidification of the paraffin. If they are not to be cut at once, the paraffin blocks are labelled (by pinning on a label). Before cutting, one pares off the paraffin up to the immediate vicinity of the imbedded tissue in order to see better in which direction it is to be cut. The piece of tissue is then fixed on the block of the microtome and cut with the knife, which is dry. The sections are placed in a bowl of water warmed to between 40–45° C., and at once smooth themselves out. They are now removed from the water by inserting clean slides beneath them and lifting them out in such

fashion that they lie smooth on the slide. The slide is then stood on end to drain off the water, and the drying is completed by placing it in the incubator at 37° C. The slide with sections attached to it is placed in the paraffin oven until the paraffin melts and begins to run off. The paraffin is then removed by xylol, and the latter removed in turn by absolute alcohol. The section sticks firmly to the slide.

4. *Freezing in oil of aniseed.* The pieces of tissue are freed as much as possible from alcohol by drying them between blotting-paper, and they are then placed for at least twenty-four hours in aniseed oil. The pieces of tissue are immersed in aniseed oil in tightly-fitting glass bottles in the incubator at 37° C., at which temperature the oil is in a fluid condition. They are then transferred with a few drops of the oil to the freezing microtome; the oil is brought to freezing-point by the ether blast, and the object is cut. The sections are thawed in aniseed oil at 37° C.; and the oil afterwards removed by placing them in absolute alcohol repeatedly renewed before staining them. Cacao butter may be used in place of aniseed oil if desired.

(*a*) *Quick method of Hardening and Imbedding according to Lubarsch* (*D. m. W.* 1903, No. 48). 1. Pieces of tissue (if possible fresh—older post-mortem material is less suitable) not exceeding 0·5 cm. in thickness are immersed in a wide test-tube in 10% formalin solution, and placed for 10–15 minutes in the paraffin oven at 50–55° C.; the formalin should be changed once or twice. 2. Place in 90–95% alcohol for 5–15 minutes;

the alcohol should be changed once. 3. 10 minutes in absolute alcohol; this should be changed twice. 4. Imbed in perfectly clear aniline oil until the pieces are quite transparent (10–30 minutes). 5. Remove the aniline oil with xylol. Change the xylol till it no longer becomes yellow (10–30 minutes). 6. Imbed in paraffin for 10–60 minutes. Procedures 2-6 should be carried out in the paraffin oven.

(*b*) *Henke-Zeller method.* Pieces of tissue 1–3 mm. thick are placed in anhydrous acetone 30-40 minutes at 37° C. and for the same time in paraffin. Duration of process 1–1½ hours.

(*c*) *Scholz' method* (*D.m.W.* 1905, No. 11). Pieces of tissue 3–5 mm. thick are placed in pure acetone 30–40 minutes at 37° C. Shake in a mixture of equal parts of absolute alcohol and ether; transfer to thin celloidin 4–5 hours in the incubator, then to thick celloidin 2-3 hours. Pour into a flat dish and dry rapidly under a bell jar by evaporation of chloroform. Specimens are ready to be cut after 12–14 hours.

For **staining,** especially for simple staining, the sections removed from the alcohol are placed 1-3 at a time in a capsule filled with the dye (a four-cornered salt-cellar is better than watch-glasses, which are easily overturned). The staining may be hastened by placing preparations in the stain in the incubator at 37° C.

The sections are next 'differentiated', the deeply stained tissue elements being cleared by means of dilute acids, or by either dilute or acid alcohol. For special details, see the individual staining methods given later. The sections, which previously were

intensely stained, thus become lighter. The next step is dehydration in absolute alcohol—a necessary preliminary to the subsequent clearing. As the sections rapidly become hard and brittle in alcohol, they must be smoothed out as much as possible before being placed in it. When immersed in alcohol, the sections are kept in the uppermost layers, these being most free of water. The next step is to clear the sections in cedar oil (not immersion oil, but ordinary cedar oil which should not be thick from old age), or in bergamot oil, or origanum oil, or oil of cloves (often absorbs much of the stain), or in xylol (rendered turbid by traces of water). At this stage the section is examined microscopically by dropping on a cover-glass and examining it in the oil. If the specimen is to be preserved, the oil is then removed and the preparation is washed with xylol and mounted in balsam. Sections cleared in xylol are not examined in xylol, as it quickly evaporates, but in balsam. The balsam sets firm in a few days. For mode of removing excess of balsam see p. 59. Sections either free, or dried on the slide, are dealt with in the same way, except that in the latter case the solutions are placed on the part of the slide where the section is attached, or the whole slide may be immersed in the solution. Sections of tissue not imbedded in paraffin can also be placed on slides, dried, and stained. For details, see under individual staining methods.

Examine sections always in the first place with a low power for general information as to the staining of the section, and also for observation of the grosser pathological changes.

For manipulating the sections one uses preferably glass needles with rounded ends made from drawn-out glass rods. Metal needles are not so good, as they tend to spoil through contact with some of the materials used in staining. If a spatula of platinum or other metal is used, it is best restricted to the transference of sections to slides.

Dyes used for Staining Bacteria.

Bacteria are generally stained by the following basic aniline dyes—gentian violet, methyl violet, dahlia, methylene blue, fuchsin, and (very similar in composition) rubin, Bismarck brown (vesuvin). These dyes stain the cell nucleus intensely, and bacteria and the other tissue elements also, though in less degree. When staining with methylene blue, much heat must not be applied; other stains, however, stand heating well.

For staining the tissue elements in contrast to bacteria, in addition to basic dyes, acid aniline dyes are used (especially eosin), which, however, stain the nucleus but feebly. As regards other dyes, carmine may be mentioned (some micro-organisms stain also with these dyes, e.g. Staphylococcus aureus). In order to avoid disappointments, one uses only dyes coming from well-known works.

Preparation of the Simplest Stains.

1. *Watery alcoholic solutions.* Saturated solutions of the dyes in alcohol at the room temperature are kept ready as stock solutions. These solutions are made by placing some of the dye in a glass-stoppered

bottle, and then adding alcohol in quantity insufficient to dissolve the whole of it. Stock solutions are not suited for staining purposes. The staining solution is prepared from them by filtering the stock solution into distilled water until a point is reached when the latter begins to become opaque. Watery alcoholic solutions keep for a long time, but old solutions stain less intensely than fresh ones.

2. *Watery solutions.* Dye is added in excess to distilled water, which is then well shaken and filtered. The solution keeps as well as in the case of No. 1.

Generally speaking, better stained preparations are obtained with dilute stain and long contact than with more concentrated stain and briefer contact.

The stronger Aniline Dyes.

The following stain more intensely than the simple aniline dyes:—

(*a*) *Loeffler's methylene blue.*

30 c.c. of saturated alcoholic methylene blue.

100 c.c. of 0·01 % caustic potash (= 1 c.c. of 1% caustic potash to 100 c.c. of water). Keeps well.

(*b*) *Aniline water stains.*

Sufficient aniline oil is added to a test-tube to cover its bottom. The test-tube is then filled three-quarters full of water and thoroughly shaken. After shaking there must still be some of the oil undissolved. The filtrate that passes through a moistened filter-paper must be as clear as water (the oil should not be poured on to the filter), and must no longer contain any oil drops. If it does, refilter. To this clear watery solution of aniline oil, saturated solution of

gentian violet, methyl violet, or fuchsin is added until a shining film appears on the surface—or one may dissolve as much of the solid dye as will dissolve. The staining power may be increased by addition of 1 c.c. of 1% NaOH per 100 of the solution. These stains keep badly, as also does the aniline water itself.

(*c*) *Carbol-fuchsin, according to Ziehl-Neelsen.*

100 c.c. 5 % carbolic acid.

10 c.c. saturated alcoholic solution of fuchsin.

When diluted 3–4 times stains more slowly, but better. Keeps well.

(*d*) *Carbol-glycerine-fuchsin, according to Czaplewski.*

Mix 1 gram of fuchsin with 5 c.c. of liquid carbolic acid. Add 50 c.c. glycerine, then 100 c.c. distilled water. Is used for staining when diluted from 4–10 times. Keeps well.

(*e*) *Carbol-methylene blue, according to Kühne.*

1·5 gram methylene blue.

10 c.c. absolute alcohol.

100 c.c. 5 % carbolic acid.

Keeps well.

(*f*) *Carbol-thionin, according to Nicolle.*

10 c.c. saturated solution of thionin in 50 % alcohol.

100 c.c. 1% carbolic acid.

Keeps well.

Simple Staining of Film-preparations.

Methylene blue, fuchsin, and gentian violet are chiefly used for this purpose (see pp. 66–67).

The choice of stain depends upon the taste of the

observer for one or another dye. Some bacteria stain better with one dye than with another (see diphtheria, p. 109; cholera, p. 142). Blood, pus, and smears from agar cultures generally stain most sharply with methylene blue, which, however, has the disadvantage of rapidly fading.

Simple Staining of Sections.

(*a*) *According to Loeffler.*

1. Stain in alkaline methylene blue solution for 5–30 minutes. Aniline water fuchsin or carbol-fuchsin may also be used.

2. Differentiate in $\frac{1}{2}$–1% acetic acid till the tissue gets clear. This takes from a few seconds to half a minute, according to thickness of the section, and intensity of the staining.

3. Dehydrate in absolute alcohol. If the alcohol becomes stained it must be changed.

4. Clear in cedar oil.

Bacilli and tissues are either blue or red.

(*b*) *According to R. Pfeiffer.*

1. Stain in dilute carbol-fuchsin (Ziehl's solution 1 + aq. dest. 3) from 15–30 minutes.

2. Transfer to absolute alcohol + 1–2 drops of acetic acid per glass capsule. As soon as the section begins to become red-violet transfer to

3. Cedar oil or xylol, &c.

(*c*) *With gentian violet.*

1. Stain in watery solution of gentian violet for $\frac{1}{4}$–$\frac{1}{2}$ hour.

2. Wash first in 50 % alcohol, then in absolute alcohol until the section has a bright violet colour.

3. Clear in cedar oil.

(*d*) *Kühne-Pregl method.*

1. Stain in carbol-methylene blue for ½–1 minute.

2. Wash quickly in water.

3. Decolorize in 50 % alcohol until the section becomes pale blue with a slight tinge of green.

4. Dehydrate in absolute alcohol.

5. Clear in cedar oil, &c.

(*e*) *Methylene-blue-tannin method of Nicolle.*

1. Stain with either alkaline methylene blue or carbol-methylene blue as under (*a*) and (*d*).

2. Wash in water or in ½–1 % acetic acid for a few seconds.

3. Transfer to 10 % tannin solution (whereby the methylene blue is rendered insoluble) for a few seconds. (1 % tannin, as a matter of fact, will do.)

4. Wash in water, dehydrate in alcohol, clear in oil, &c. This method may be recommended for bacilli readily decolorized, such as typhoid, glanders, &c.

(*f*) *Nicolle's thionin stain.*

1. Stain in thionin solution for ½–1 minute (10 parts of saturated solution of thionin in 50 % alcohol + 100 parts of 1 % carbol water).

2. Wash in water.

3. Decolorize in absolute alcohol, then pass through oil, &c.

This method, like (*e*), is recommended for bacteria that easily part with their stain.

The methods (*a*), (*b*), and (*f*) are to be preferred because of their greater simplicity.

Staining of the Bacteria alone, or Contrast Staining.

I. *The method of Pick-Jacobson.* For film preparations. Stain for 8–10 seconds at the utmost in a mixture of Ziehl's carbol-fuchsin (p. 68) 15 drops, saturated alcoholic methylene blue 8 drops, aq. dest. 20 drops.

The bacteria stain dark blue, nuclei bright blue, the rest of the tissue red. (N.B.—If old, this stain is improved by the addition of a little carbol-fuchsin.)

II. *Eosin and methylene blue.*

For film preparations.

1. Stain for half a minute in a freshly-made mixture of Loeffler's methylene blue (p. 69) 30 parts, and saturated alcoholic solution of eosin 10 parts.

2. Wash in water.

Bacteria and nuclei stain blue, cell protoplasm, &c., red.

III. *May Grünwald's method* (*Zentralbl. f. inn. Med.*, 1902, No. 11).

Several litres of 1 : 1000 watery solution of eosin are mixed with the same quantity of a solution of methylene blue (medicinale, Höchst), of similar strength, and after a few days filtered. The portion that remains on the filter is washed with water until this passes away almost colourless. From this precipitate, when dry, a saturated solution in methyl alcohol is prepared and used for staining. The film preparations are not fixed. They are stained in the cold for a few minutes up to as many hours, and are then washed in water containing a few drops of the solution. The stain can be

obtained from Dr. Schwalm, München, Sonnenstr. 10. It is especially used for preparations of blood and pus. Assmann (*M. m. W.* 1906, No. 28) lays his preparations in Petri dishes, pours over them forty drops of the solution for three minutes; adds thereupon 20 c.c. of distilled water + 5 drops of 1 : 1000 K_2CO_3, shakes, and allows the stain to act for five minutes; then, without washing, dries, &c.

IV. *Weigert's stain.* For sections.

1. Stain in watery alcoholic gentian violet for 5–10 minutes.
2. Wash in alcohol for 5–10 seconds.
3. Wash in water for five seconds.
4. Counter-stain in picrocarmine (p. 78) for 1–12 hours.
5. Wash in alcohol.
6. Clear in cedar oil, &c.

Bacteria stain blue, cell nuclei red, rest of tissue red to yellow. This stain is not to be recommended for bacteria that are easily decolorized, e.g. typhoid, glanders, fowl cholera, &c.

V. Gram's Stain.

Since only certain kinds of bacteria are stained by it, Gram's stain is used not only for the demonstration of bacteria, but also for diagnostic purposes.

(*a*) *For film preparations.*

1. Stain with warm aniline water gentian violet, or aniline water methyl violet (p. 68), for at least two minutes. (The best stains are methyl violet, Höchst 6 B and BN.)

1 *a*. Wash in aniline water. (This may be omitted.)

2. Place for thirty seconds to two minutes in iodine and potassium iodide solution. (This solution is made up as follows:—iodine 1 part, potassium iodide 2 parts dissolved in aq. dest. 5 parts. After solution, add aq. dest. 295 parts.) When placed in this solution there develops in the bodies of certain kinds of bacteria (see p. 75) a combination between the stain and iodine that is insoluble in alcohol.

3. Decolorize the preparation in absolute alcohol until no more colour is given off. Those bacteria that are capable of being stained by Gram are now found to be stained blue-black—all other bacteria and the tissue elements (excepting some of the cell nuclei) are unstained. The preparation can at this point therefore be examined in water, or it may be dried and mounted in balsam.

4. Counter-stain with watery alcoholic solution of safranin or fuchsin for several seconds; or with picrocarmine (p. 78) for 2–10 minutes. Counter-staining with fuchsin has the advantage that any bacteria present in the preparation that may have parted with the violet stain are much better stained with it than with safranin or carmine.

5. Wash in water.

Bacteria blue-black; tissue red.

(*b*) *Sections* (see remarks under (*a*)).

1. Stain in aniline water gentian violet or in aniline water methyl violet for 5–30 minutes.

1 *a*. Wash in aniline water for thirty seconds. (This may be omitted.)

2. Transfer the preparation to the potassium iodide

solution for 1–2 minutes. The sections here become dark brown.

3. Wash in absolute alcohol until the section appears entirely or partly unstained. At this stage the bacteria positive to Gram alone are stained, and are blackish-blue in colour. The preparation may then be either cleared in cedar oil, &c., or it may be counter-stained in order to bring out the tissue and bacteria which have not stained by Gram.

4. Wash in water, and then counter-stain with picrocarmine (p. 78) or safranin, or with dilute fuchsin, for 5–10 minutes.

5. Wash in 60% alcohol.

6. Dehydrate in absolute alcohol.

7. Clear in cedar oil, &c.

If desirable, one may carry out 4 and 5 first, and then 1–3 followed by 7.

Gram-positive bacteria blue-black, tissue red (cell nuclei often pale blue to dark blue). Bacteria are frequently not stained equally well in all parts of the preparation.

Modifications of Gram's method.

(*a*) Instead of making up the stains with aniline water, one can use 1–2½% of carbol water, in which case they keep longer. After staining, the preparations should be washed in carbolized water of corresponding strength. The addition of one-tenth part of a saturated alcoholic solution of methylene blue to the staining solution is also to be recommended.

(*b*) For accelerating decolorization according to Günther, alcohol absolute + 3% HCl may be used for ten seconds followed by absolute alcohol; and accord-

ing to Nicolle, alcohol absolute +20–30 vols. per cent. of acetone. (Nicolle recommends carbol water gentian violet made up with 1% carbol water, and potassium iodide solution of the following composition:—1 K + 2 Ki + 200 Aq.)

The following micro-organisms stain by Gram's method, i.e. continue to remain blue-black after treatment of the preparation with alcohol:—Anthrax, tetanus, tubercle, leprosy, diphtheria, swine erysipelas, mouse septicaemia, the pyogenic streptococci and staphylococci, Fraenkel's pneumococcus, Micrococcus tetragenus, Streptothrix actinomyces, yeasts, potato bacillus, and others. The stain, however, is only useful for differentiating bacilli such as tetanus, tubercle, or leprosy, when it is certain that there are no other Gram-positive bacilli present as well in the tissue. If the treatment with alcohol is prolonged too far, some of the above Gram-positive micro-organisms become decolorized.

The following micro-organisms do not stain by Gram's method, i.e. part with their stain when the preparation is treated with alcohol:—Typhoid, coli, and similar bacilli. Cholera and similar vibrios, fowl cholera, and rabbit septicaemia. B. malignant oedema, and the bacillus of quarter evil or rauschbrand (the latter two are said to occasionally remain positive to Gram), Friedlander's bacillus, plague, glanders, influenza, spirillum of relapsing fever, gonococcus, and meningococcus.

When examining the reaction of a micro-organism to Gram's stain one always uses a young culture, and as a control puts on a part of the preparation in the case

of bacilli a smear from a young Staphylococcus aureus culture, or in the case of cocci a smear from a young anthrax culture. Then, if the stain is applied correctly, the aureus or anthrax are stained blue-black. The treatment with alcohol must not be too short, because in that case all bacteria will be found to be stained. If desirable, the control may be carried further by also putting on part of the preparation a smear from a young culture of a micro-organism negative to Gram's stain, e.g. coli, typhoid, or cholera.

VI. *Weigert's stain for sections*—the so-called Fibrin method.

1. Stain in lithium carmine (carmine 2·5–5 dissolved in saturated watery solution of lithium carbonate 100) or in watery alcoholic solution of safranin, for $\frac{1}{4}$–$\frac{1}{2}$ hour. If desirable, a stain composed of Ziehl's carbol-fuchsin 1 + aq. dest. 4 may be used instead.

2. Wash in 50% salt solution.

3. Stain in aniline water gentian violet for 5–30 minutes—or according to Kühne the following stain is preferable: crystal violet 1, dissolved in absolute alcohol 10; of this 1 + aq. dest. 10 + 2 drops HCl.

4. Wash in 0·6% salt solution.

5. Transfer the section to a slide and dry it with blotting-paper.

6. Place it in potassium iodide solution for 1–2 minutes.

7. Dry with blotting-paper.

8. Decolorize in aniline oil till the oil becomes no longer stained. The staining of the section is controlled by examining it microscopically.

9. Remove the aniline oil with xylol.

10. Canada balsam.

The bacteria are stained violet blue, fibrin deep blue, tissue red. Picrocarmine is not particularly well suited for the preliminary staining of the tissue, since the aniline oil extracts the picric acid from the tissue.

VII. *The stain of Claudius.* This method yields the same result as Gram's method (the picric acid acts like the potassium iodide solution), gives less precipitate than Gram, and stains the bacilli of malignant oedema and rauschbrand blue-black.

(*a*) *For films.*

1. Stain in 10% watery solution of methyl violet for one minute, then wash in water and dry with blotting-paper.

2. Wash in saturated watery solution of picric acid + aq. dest., equal parts.

3. Wash in water, dry with blotting-paper.

4. Wash in chloroform or oil of cloves till the preparation is decolorized.

5. Dry with blotting-paper, mount in balsam.

(*b*) *For sections* (best carried out on the slide).

1. Stain as above for two minutes.

2. Wash in water, dry with blotting-paper.

3. Picric acid as above for two minutes.

4. Wash in water, carefully dry with blotting-paper.

5. Treat with oil of cloves till the section is decolorized.

6. Xylol, Canada balsam.

Bacteria blue; tissue unstained or yellowish, but if first counter-stained with carmine can be rendered red as in case of Gram.

Picrocarmine for counter-staining tissue in the methods of Gram, Weigert, or Claudius.

(*a*) *Prepared according to Friedlander.*

Dissolve carmine 1 in aq. dest. 50 + ammonia 1. Add saturated watery solution of picric acid until the precipitate that forms no longer dissolves on shaking. The addition of a little ammonia will redissolve the precipitate. To prevent growth of bacteria in the stain a few drops of carbolic are added. Filter before use. The stain will keep.

(*b*) *Prepared according to Weigert.*

Allow carmine 2 parts + ammonia 4 parts to stand for twenty-four hours. Then add 200 c.c. of concentrated watery solution of picric acid. Twenty-four hours later add acetic acid drop by drop till a precipitate forms, then ammonia drop by drop till the solution is clear.

Capsule-staining.

In cover-glass preparations many bacterial capsules take the stain when the preparation is warmed for a more or less long time in Loeffler's or Ziehl's stains (pp. 67, 68): the capsules in the former case staining blue and in the latter red. Among special methods for staining capsules are the following :—

(*a*) *Friedlander's method for films.*

1. Dip the cover-glass for 1–3 minutes in 1% acetic acid.

2. Drain off the acetic acid and quickly dry.

3. Stain for a few seconds in saturated aniline water gentian violet.

4. Wash in water and examine therein. In case of the capsule being stained so deeply as to obscure the body of the bacteria :—

5. Wash in 1% acetic acid or 50% alcohol for ten seconds.

6. Wash in water, examine therein, and if the stain is successful, dry, and add balsam.

For sections.

1. Stain for twenty-four hours at 37° C. in concentrated alcoholic gentian violet 50 parts + aq. dest. 100 + glacial acetic acid 10.

2. Differentiate in 1% acetic acid.

3. Dehydrate in alcohol, oil, &c.

When 2 is not sufficiently carried out, the capsules are stained as intensely as the bacterial bodies and hide them.

(*b*) *Weidenreich-Hamm method.*

Rub up some of the preparation in a drop of serum. Fix with osmic acid for not more than 30–40 seconds, stain with Giemsa's stain (see Malaria).

(*c*) *Johne's method.*

1. Stain with warm 2% aqueous gentian violet for 1–2 minutes.

2. Wash in water.

3. Differentiate in 1–2% acetic acid for 6–10 seconds.

4. Wash in water and examine therein. Canada balsam causes the capsules to fade.

(*d*) *Ribbert's method* (*for films only*).

1. Stain the cover-glass for a few seconds in the following solution :—Aq. dest. 100, alcohol abs. 50, glacial acetic acid 12·5 + dahlia as much as dissolves on heating.

2. Wash in water and examine therein, or dry and mount in balsam. The latter renders the capsules less distinct.

(*e*) *Bunge's method for staining flagella* (p. 84).

(*f*) *Nicolle's method for films or sections.*

1. Stain with the following mixture:—Saturated solution of gentian violet in 95% alcohol 10 parts + 1% carbolic water 100 parts.

2. Wash in absolute alcohol + $\frac{1}{3}$ its volume of acetone. Then in case of cover-glass preparations wash in absolute alcohol, or in case of sections pass through oil, &c.

(*g*) See for further methods under anthrax bacillus, p. 89; these are also applicable for several other bacteria.

Spore-staining.

(*a*) 1. Stain the cover-glass preparation in aniline-water fuchsin or in carbol-fuchsin, heating strongly. (Stain for one hour if the preparation is floated on the stain film downwards, or for ten minutes if the stain is dropped on to the surface of the cover-glass, adding fresh stain to replace any lost by evaporation.)

Better penetration of stain into the spores is effected if the preparation is passed thirty or forty times through the flame in the fixing.

2. Wash in alcohol + 3% HCl (or 1% H_2SO_4, or 3% HNO_3) for $\frac{1}{2}$–1 minute.

3. Counter-stain in watery alcoholic solution of methylene blue.

4. Wash in water and examine therein, or dry and mount in balsam.

Spores red, bacilli blue.

(*b*) Preferable because more reliable is the method of Möller.

1. After fixing it, place the preparation for from five seconds to ten minutes in 5 % chromic acid. The exact time of exposure for each micro-organism must be found by experiment.

2. Wash in water.

3. Place the preparation in aniline-water fuchsin or in carbol-fuchsin and heat to boiling-point.

4. Decolorize for five seconds in 5 % H_2SO_4.

5. Wash in water.

6. Counter-stain with methylene blue.

Prior to treatment with chromic acid the preparation may be placed for two minutes in chloroform to remove fat-drops, &c., which may simulate spores; the cover-glass is then washed in water.

Instead of treatment with chromic acid, the spore membrane may be macerated in a concentrated solution of iodine and zinc chloride, or in a solution of hydrogen peroxide, though both are less active than chromic acid.

According to Aujeszky (*Ctrbl. f. Bakt.*, Abt. I, Bd. 23, p. 329), the air-dried unfixed film preparation should be placed for 3–4 minutes in hot $\frac{1}{2}$ % HCl, washed in water, dried, fixed, and then stained as in 3–6 above.

According to Orszag (*Ctrbl. f. Bakt.* I, Or. 41, p. 397), one rubs up the material with a drop of a mixture of four parts of $\frac{1}{2}$ % sodium salicylate solution and one

part 5% acetic acid, dries in the air, fixes in the flame and treats as in (*b*) 3–6, except that one uses 1 % H_2SO_4 or 3 % HNO_3.

Flagella-staining.

For the observation of flagella in living bacteria see Dark Ground Illumination, p. 6.

Having seen that the organism exhibits definite motility in the hanging drop, a portion of its growth on some solid medium is taken for flagella-staining. Fluid media should not be used ; a young agar culture is especially suitable. The bacteria should lie as separate as possible on the cover-glass, and the film should contain as little of the nutrient material as possible. This may be effected in the following way : – A droplet of water is laid upon each of six cover-glasses, which must have been most carefully cleaned. Some growth from a culture is transferred to the first drop, from this a trace is transferred to the second, from this to the third, and so on. Another way is to add a little bacterial growth to a droplet of water on a slide, and to transfer from this a trace to a larger drop of water to which 1–2 loops of 2 % osmic acid solution have been added. From this drop one prepares cover-glass preparations. A third method is to make a thin suspension of the bacteria in 0·8 % NaCl solution in a test-tube, to transfer droplets from it to cover-glasses, and spread them out without rubbing. The cover-glasses are allowed to dry in the air and then fixed by passage through the flame.

The cover-glasses must always be absolutely clean. (For method of cleaning see p. 8.)

A. Loeffler's method of Flagella-staining.

1. Place some of the mordant described below upon the cover-glass and warm till steam arises.

2. Carefully remove the mordant by washing in a stream of water. If necessary use blotting-paper.

3. Wash in alcohol until only those parts where the bacteria lie appear stained.

4. Stain in aniline water fuchsin and apply heat. The fuchsin solution is prepared according to the directions given on p. 67, and to it is added 1 % of a 1 % or stronger solution of caustic soda until a precipitate begins to form—i. e. the solution begins to become turbid.

5. Wash in water.

The mordant consists of the following mixture :— 10 c.c. of a 20 % solution of tannin, 5 c.c. of cold saturated solution of ferrous sulphate, 1 c.c. of a watery or alcoholic solution of either fuchsin or methyl violet.

According to Loeffler, this mordant is exactly suited for demonstrating the flagella of Spirillum concentricum. For typhoid, an addition of 1 c.c. of a 1 % solution of caustic soda to 16 c.c. of the mordant is necessary, for B. subtilis 28–30 drops, and for B. mal. oed. 36–37 drops of the caustic. On the other hand, $\frac{1}{2}$–1 drop of a solution of H_2SO_4 (exactly equivalent to the 1 % solution of caustic soda) must be added to 16 c.c. of the mordant in the case of cholera and Spirillum rubrum.

According to Nicolle and Morax, neither caustic nor acid need be added if the preparation is treated in the mordant for three or four times in succession

for ten seconds each time until steam is given off (not boiling), and carefully washed in water between each application of the mordant. Instead of being washed in alcohol as directed in 3 above, the preparation may be washed in water.

The formation of precipitate on the preparation can be avoided by very careful washing. To this end also it is practicable when applying the mordant or stain to cover the film with a small piece of blotting-paper, and to apply the fluid on the top of it.

Bunge's modification.

1. The following mordant is applied:—

3 parts concentrated watery solution of tannin, 1 part liquor ferri sesquichlor 1:20 aq.; to 10 c.c. of this mixture add 1 c.c. of a concentrated watery solution of fuchsin. This mordant should be kept for at least several days before use. Immediately before use peroxide of hydrogen is added drop by drop till the mordant assumes a reddish-brown colour (about 14 drops of 3% H_2O_2 solution to 5 c.c. of the mordant are required). A few drops of the solution are then filtered on to the cover-glass and the latter warmed over the flame for five minutes. When H_2O_2 has been added to it, the mordant keeps for only a short time.

2. Wash in water.

3. Dry between blotting-paper.

4. Stain with carbol gentian violet (prepared in the same way as Ziehl's solution, p. 74 (*a*)), and heat gently.

5. Place for $\frac{1}{2}$–1 minute in 1 % acetic acid. (May be omitted.)

6. Wash in water, dry, mount in balsam.

The above method can be applied to all bacteria without any addition to the mordant. Capsules are stained by it, especially if before being subjected to the mordant the preparation is placed for $\frac{1}{2}$–1 minute in 5 % acetic and then washed in water.

The Modification of Coerner and A. Fischer.

1. Place the preparation in the following mordant, and heat for one minute without boiling:—Tannin 2 grams, water 20 grams, solution of ferrous sulphate of 1 : 2 strength 4 c.c., saturated alcoholic solution of fuchsin 1 c.c.

2. Wash in water.

3. Stain with aniline-fuchsin, or with carbol-fuchsin, or with saturated watery solution of fuchsin.

4. Wash in water. Dry. Mount in balsam.

This method is applicable for all bacteria without any addition to the mordant.

B. Peppler's Method (*Ctrbl. f. Bakt.* I. 29, p. 345).

1. Place 1–5 minutes in the following mordant without warming:—

Dissolve 20 grams of tannin in 80 c.c. distilled water with gentle warming in a water-bath, cool to about 20° C., add slowly in small quantities with shaking 15 c.c. of a 2·5 % solution of chromic acid (free from sulphuric acid) in distilled water; leave to stand 4–6 days at room temperature (not below 18° C.) filter through a double filter avoiding cooling. Preserve at room temperature, filter before use.

2. Wash in a stream of tap-water, drain thoroughly.

3. Stain 2 minutes with carbol gentian violet solu-

tion without warming (10 c.c. 5 % alcoholic gentian violet solution, liquefied carbolic acid 2·5 c.c., distilled water to 100 c.c.; the solution to be left standing for a few days and filtered without shaking).

4. Wash thoroughly with water, dry, and mount.

C. Van Ermengem's Method.

1. Place the cover-glass preparation for half an hour in the cold, or for five minutes at a temperature of 50–60° C., in a mixture of 1 part 2 % osmic acid and 2 parts 10–25 % tannin solution, to which mixture 4–5 drops of glacial acetic acid are added per 100 c.c. The mixture should, if possible, be a few days old.

2. Wash in distilled water.

3. Wash in absolute alcohol.

4. Immerse in 0·25–0·5 % silver nitrate for a few seconds.

5. Without washing, place for a few seconds in the following solution:—Gallic acid 5·0, tannin 3·0, fused acetate of soda 10·0, aq. dest. 350 c.c.

6. Place the preparation back in 4, and keep it constantly moving till the solution begins to become black.

7. Wash in much water. If the staining is not sufficiently intense repeat 5–7; but if too dark, immerse in chloride of gold 1 : 3,000 for a moment, carefully wash, and expose the preparation for a few days to the light.

8. Dry with blotting-paper; mount in balsam.

Bacteria blackish-brown; flagella black.

The method may be applied to all motile bacteria

in the same manner. Best carried out in bright daylight.

D. Zettnow's Method (*Klin. Jahrb.*, Bd. xi, p. 379 ; older communication *Zschr. f. Hyg.*, Bd. xxx, p. 95).

1. Preparation of the film by the osmic acid method (see p. 57).

2. Mordant. The cover-glasses are fixed in the flame, and laid face downwards in a 'block capsule', which is well filled with the mordant and placed for 5–7 minutes on an iron plate which is at a temperature of about 100° C.

Preparation of the mordant. Make a solution of 10 parts tannin in aq. 200, and warm it up to 50–60° C. Add 36–37 c.c. of a solution of 2 grams tartarus stibiatus (antimony tartrate) in 40 of water, and heat till the precipitate dissolves. If, on cooling, the mordant is very turbid (milk-white; test by pouring it into a test-tube) add some tannin solution ; but if clear, add 1 c.c. of tartrate solution. The mordant should on no account form any precipitate, and it should be quite clear on heating. The addition of thymol ensures its preservation. The mordant should be applied warm and in clear condition.

3. Allow the capsules to cool down until the mordant begins to become turbid, then wash carefully with water.

4. On the cover-glass put 3–4 drops of ethylamine-silver solution, and heat until it fumes well and the edges of the film (these alone) become blackened.

Ethylamine-silver solution. 2–3 grams of silver sulphate are thoroughly mixed with 200 aq., in order

to obtain a saturated solution. The desired quantity of this, together with an equal volume of water, is put into a test-tube and 33 % ethylamine solution, added until the precipitate which forms is dissolved. The solution keeps well. The brown tinge which it gradually acquires is of no importance.

VI

Special Staining and Culture Methods for the most important Pathogenic Micro-organisms and a few others

1. The Anthrax Bacillus.

B. anthracis grows on all ordinary culture media, liquefies gelatine, stains easily, and is Gram-positive. It is non-motile. Spore-formation occurs most quickly in the hot incubator, but does not take place below 16° C., or in the animal body (when whole).

The presence of B. anthracis in the body is proved by culture and by animal experiment (subcutaneous injection of mice and guinea-pigs). In the case of decomposed material the animal experiments sometimes fail, while plate cultures succeed. In the blood of infected animals which cannot be examined at once, the anthrax bacilli retain their vitality for some considerable time if the blood is dried in thick layer on glass. For demonstration of anthrax on hair, &c., wash the hair in alkaline broth and heat for

half an hour to 80° C. (the anthrax spores survive), centrifugalize, and inoculate the sediment into animals and plates.

Capsules: the capsules may be stained by the methods given.

The capsules may also be demonstrated by warming the preparation in a solution of Safranin (three grams of Safranin dissolved in 100 c.c. of almost boiling aq. dest.: when cooled, the solution is filtered). Another method of demonstrating capsules is by staining with gentian violet (a cold, saturated solution of the dye in formalin; filtered). In this case the smear preparations are not fixed, and are subjected to the stain for thirty seconds in the cold. The preparations are examined in water.

The possession of a capsule sometimes enables B. anthracis to be distinguished from bacilli that resemble it in appearance, and are apt to occur in the animal body when decomposition has begun; but positive cultures and animal experiments are always also necessary before a definite decision can be given.

The Preparation of Spore-threads.

Agar, or potato cultures in which the presence of spores has been proved by microscopical examination are broken up and emulsified in sterile water. Sterile silk threads (No. 4 or 5) 1–2 cm. long are soaked in this emulsion (or are rolled directly on the agar culture). They are then dried on sterile plates in the exsiccator, and preserved in sterile test-tubes in the dark. Handle the threads with care, owing to the

danger of spore inhalation. The use of these threads for testing disinfectants is described on p. 53. As a rule they withstand steaming at 100° C. for 2–5 minutes.

2. The Tubercle Bacillus.

The usual media for B. tuberculosis are egg media (see p. 97), blood serum, glycerine agar (optimum 4 % glycerine), glycerinated potato (wedges, see p. 28, are boiled in water containing 4 % glycerine, and the fluid then poured away), glycerine broth (growth occurs only in the form of particles floating on the surface). Cultures must be kept at a temperature of 37° C., and growth is very slow. Drying up of the medium is prevented by covering the culture tubes with a rubber cap. There is practically no growth on gelatine, peptone-agar, &c., or at room temperature. The tubercle bacillus is non-motile.

Special media, giving good growth already after eight days, are Hesse's Heyden-agar, p. 94, or Ficker's brain medium. The latter is prepared as follows:—

Finely chopped brain is mixed with an equal part of aq. dest., and warmed to boiling, constantly shaking. The mixture is boiled for a quarter of an hour, then strained to a thin pulp. With this is next mixed, without neutralizing, an equal part of 2·5 % agar dissolved in aq. dest.; 3 % of glycerine is then added and the whole sterilized. When the medium is poured into tubes, the tubes should be well shaken, and then set quickly before the layers of brain and agar have time to separate.

Methods of Staining the Tubercle Bacillus.

Tubercle bacilli stain with difficulty, but when once stained retain the stain very firmly. The following methods demonstrate only tubercle bacilli, and similar so-called acid-fast bacilli. As regards differentiation of the latter from tubercle bacilli, see p. 101. In these processes the tubercle bacilli take the primary stain, whilst other bacteria and the tissue elements take the counter-stain.

(*a*) The following two staining methods are most to be recommended:—

For films.

1. Stain with aniline water fuchsin, or with carbol-fuchsin for two minutes, boiling repeatedly.
2. Decolorize for 2–5 seconds in 5 % H_2SO_4, or in 25 % HNO_3.
3. Wash in 70 % alcohol till the film appears colourless (if this does not occur quickly, repeat 2 and 3).
4. Counter-stain with either saturated watery solution of methylene blue, or with Loeffler's blue (p. 67), 1 part and 3 parts of water for 5–10 seconds.
5. Wash in water.

For sections.

1. Stain in aniline water fuchsin (carbol-fuchsin is not so good, because the preparations are less clean) for from fifteen minutes to twenty-four hours.
2. Decolorize for ten seconds in 5 % H_2SO_4, or in 20 % HNO_3.
3. Wash in 70 % alcohol till the section appears

colourless (in order to hasten decolorization, 2 and 3 may be repeated).

4. Counter-stain with methylene blue—preferable Loeffler's blue 1 part to 3 parts of water.

5. Wash in $\frac{1}{4}$–$\frac{1}{2}$ % acetic acid.

6. Dehydrate in absolute alcohol.

7. Clear in cedar oil, &c.

Tubercle bacilli, red; other bacteria and tissue elements, blue. Fixation by formalin diminishes the staining properties of the bacilli.

For film preparations, as also for sections, aniline water gentian violet may be used in 1. In that case, in 2 one uses 25 % HNO_3, and in 4 a weak solution of either fuchsin, safranin, or Bismarck brown. The tubercle bacilli are thus stained blue, and the tissue red or brown.

(*b*) *Method of Fränkel and Gabbet.*

For films.

1. Stain in boiling aniline water fuchsin, or carbol-fuchsin for two minutes.

2. Decolorization and counter-staining are both effected at the same time in a mixture of alcohol 30, aq. 50, HNO_3 20, and as much methylene blue powder as will dissolve. An alternative solution consists of H_2SO_4, 10 parts, aq. dest. 30, and methylene blue powder to saturation-point.

3. Wash in water.

An alternative method is as follows:—

1. Stain in boiling aniline water gentian, or methyl violet for two minutes.

2. Decolorization and counter-staining are effected

simultaneously by placing the preparation for $1\frac{1}{2}$–2 minutes in a mixture of alcohol 70 parts, HNO_3 30, and as much Bismarck brown as will dissolve.

3. Wash in water.

The disadvantage of method (*b*) as compared with (*a*) is that in (*b*) all stages of the staining and counter-staining cannot be followed with the eye. Moreover, smegma bacilli occasionally appear stained in (*b*), so this method must not be used for tubercle diagnosis in case of material liable to contain smegma bacilli.

Examination of Sputum for Tubercle bacilli.

(These directions apply also for the examination of exudation, urine, milk, &c.)

A. Microscopical examination. In the first place, film preparations are made and examined microscopically. The sputum is spread out on a black lacquered plate, or in a glass capsule which rests on a dark background, and a nodule or so-called 'lentil' is picked out and distributed on a cover-glass or slide by means of a platinum loop, or else it is flattened out between two cover-glasses or slides. Urine, or cerebrospinal fluid withdrawn by lumbar puncture, are centrifugalized, and preparations made from the sediment. The staining process to which the preparation is now subjected can only be regarded as successful when (with the exception, perhaps, of some slight staining of the epithelial cells) nothing exhibits the stain used for demonstration of tubercle but the tubercle bacilli themselves.

If, in spite of the examination of several preparations, no tubercle bacilli are found, then one resorts to

B, the augmentation method, or to *C*, the animal experiment.

B. Augmentation Methods. The following are chiefly used.

1. *Biological Methods.* Cultivation of tubercle bacilli on suitable media.

a. Hesse's method of cultivation on Heyden-agar.

Dissolve 5 grams of nutrient Heyden in 50 c.c. aq. dest., shaking well. Add this mixture to a solution composed of NaCl 5 grams, glycerine 30 c.c., agar 10–20 grams, normal soda solution 5 c.c. in aq. dest. 950 c.c., and boil for fifteen minutes, constantly shaking. Filter in the steamer, and after sterilizing the filtrate, fill it into Petri dishes, and when solidified, distribute over its surface either a 'lentil' of sputum which has been washed repeatedly in sterile water, or fine flakes of the sputum. At 37° C. tubercle bacilli multiply on this medium very quickly; being already numerous after 6–7 hours, and forming colonies visible to the eye after 2–7 days; while development of the other sputum bacteria is much impeded. The plates should be protected from drying by placing them in a moist chamber. They should be examined by means of impression preparations. The method is a very good one for quick diagnosis in case of sputa poor in tubercle bacilli. Should it be desired to obtain a pure culture, the sputum may be mixed with five times its volume of nutrient Heyden solution without the addition of agar, and incubated for twenty-four hours at 47° C., under which circumstance the tubercle bacilli multiply, and can be treated according to one of the sedimentation methods described below

(Jochmann). For Spengler's modification, in which preceding treatment with formic aldehyde is used, see *Zeit. f. Hygiene*, Bd. xlii, p. 90.

b. Hesse's method of cultivation on glycerin-water-agar. (*Centr. f. Bakt.*, Or.-Bd. xxxv, p. 386.)

Agar 1 part, glycerine 3, aq. dest. 96 parts are boiled together and filtered into test-tubes (Schott, Jena) which do not give off alkali. Each test-tube receives 20 c.c. of the filtrate, and to their melted contents just before use is added $\frac{N}{10}$ alkali until the reaction of the medium to red litmus-paper exactly corresponds with that of the sputum under investigation. The contents of these tubes are then poured into Petri dishes, and on the surface of the solidified agar a portion of the sputum about the size of a 'lentil', and as free as possible of mouth bacteria, is broken up and spread out over a wide area. After incubation for 1–2 days at 37° C., tubercle bacilli can be demonstrated by means of impression preparations.

c. Uhlenhuth's 'antiformin' method. (*Arbeiten aus dem Kaiserl. Gesundheitsamte*, 32, p. 158.)

20 c.c. of sputum are mixed with 65 c.c. sterile distilled water and 15 c.c. antiformin in a glass dish provided with a lid and standing on a dark background. After 1–2 hours the sputum becomes homogeneous, more quickly when stirred. By means of a sterile pipette 10 c.c. of the mixture are removed, centrifuged and the supernatant fluid poured off. The residue is stirred up with 10 c.c. sterile 0·8% salt solution and centrifuged, and this washing is again repeated. Four or five loopfuls of the residue are thoroughly rubbed into

the surface of half a dozen glycerin-serum tubes and incubated.

For film preparations a single washing suffices, the film is made to adhere to the slide or coverslip by means of a thin film of egg albumen or untreated sputum. Only the tubercle bacilli survive in the sediment, other bacteria are killed and dissolved.

Antiformin is a mixture of solutions of sodium hypochlorite and caustic soda, and can be obtained from Oskar Kühn, Berlin C 25, Dircksenstrasse 20, or from English dealers.

d. Antiformin. Egg medium, or animal tissue medium.

[The following procedure recommended by Cruickshank (*B. M. J.* Nov. 9, 1912) appears to be far more successful than the preceding methods.]

Method.—To the sputum, sediment from urine, or other fluid a 15 to 20 % dilution of antiformin is added, and the mixture allowed to stand, solution being aided by occasional vigorous shaking. The resulting mixture in any case should not contain more than 15 % antiformin. (This is not necessary if the sediment is to be used only for microscopical purposes.) As a rule, with the majority of sputa one volume is readily dissolved by three or four volumes of antiformin, but the amount of antiformin added to any particular specimen depends on the consistency of the sputum. Complete solution can usually be obtained within a comparatively short time, depending on the nature of the material under treatment. One or two hours is usually sufficient for sputa, but longer may be necessary with more refractory material such as thick caseous matter. In the

case of solid tissues such as tuberculous lung or gland the material is cut up into small fragments and rubbed up in a mortar with a small amount of antiformin; the latter is then poured off and the material again treated. In this way by repeated rubbing with small amounts of antiformin, the solution of a considerable amount of material can usually be obtained in a comparatively short time. Efficient antiformin should always produce distinct liberation of gas during the process of causing solution. The antiformin mixture is then centrifugalized and the sediment which results is washed twice with sterile water or saline solution. A microscopical preparation should then be made as a control to estimate the number of bacilli present in a loopful of sediment. The washed sediment is inoculated on various culture media, of which plain egg (Dorset), glycerine egg (Lubenau), and animal tissues (Frugoni) have been found to be by far the most satisfactory. As will be referred to later, it is probably advisable in each case to use all three media.

Egg Medium. – This has been prepared as follows: Two or three absolutely fresh eggs are washed and the shells sterilized by washing with undiluted formalin. The shells are allowed to dry and the eggs broken into a sterile dish. The yolks and albuminous portions are thoroughly mixed by gentle stirring (frothing is to be avoided), and the mixture is strained through sterile gauze. The volume is measured, and to three parts of egg one part of sterilized 0·85 % NaCl solution is added. In the case of glycerine egg, one part of 6 % glycerine in 0·85 %

NaCl solution is added to three parts of egg. A few drops of alcoholic basic fuchsin (sufficient to give a distinct pink colour) are added, and the mixture placed in sterile tubes. (The addition of a colouring agent is of distinct advantage in aiding the detection of early or very scanty growth.) The tubes are then placed in the sloped position high up in the steam sterilizer and coagulated (three to five minutes). Care is necessary at this stage not to expose the tubes for any longer period than is absolutely necessary for coagulation, otherwise bursting of the medium results. A smooth surface should be obtained. The tubes are further sterilized by heating at 105° C. for twenty minutes on two successive days, all the valves of the autoclave being tightly screwed down before heating is commenced. To prevent drying of the medium on standing, glycerine bouillon may be added, and a small amount should be allowed to remain in each tube at the time of inoculation. The presence of moisture in the cotton-wool stoppers must, as far as possible, be avoided, otherwise, owing to the length of time during which the tubes are incubated, moulds may grow. After inoculation the tubes should be sealed with paraffin wax. Uninoculated medium is best kept in the ice-chest.

Animal Tissue Media.—This is prepared by Frugoni's method. Fresh rabbit lung or other tissue, after soaking for an hour in 0·85 % NaCl solution, containing 6 % glycerine, is sterilized by autoclaving at 120° C. for half to three quarters of an hour, and is then supported over the surface of 6 % glycerine bouillon, so that the tissue surface is kept moist

by capillary attraction and by condensation. Tissues obtained at *post-mortem* examinations may also be used ; these are sterilized by boiling or by autoclaving. C. states that the preliminary treatment with glycerine salt solution is not necessary.

2. *Sedimentation methods.*—In these the sputum is liquefied, as a result of which the tubercle bacilli either deposit, or may be thrown down by the centrifuge. The method only affords microscopical evidence of tubercle, and is not used for purposes of culture.

a. The Method of Biedert-Mühlhauser Czaplewski.

The sputum is well shaken up with two to four times its volume of 0·2 % NaOH in a cylindrical vessel with rubber stopper for one minute. If the sputum is not equally liquefied throughout, add more NaOH and shake again thoroughly till the fluid is homogeneous. Now heat to boiling while stirring in a porcelain dish. Add 1–2 drops of phenolphthalein solution, shake thoroughly, and then add 5% acetic acid drop by drop until the red colour disappears (no more). Then add to the fluid double its volume of 96% alcohol and either allow it to precipitate in a conical grass, or centrifugalize. The deposit is used for making stained preparations.

b. Dahmen's Method.—The sputum is heated for fifteen minutes in the steamer (simultaneous sterilization): it is allowed to precipitate or it is centrifugalized; the precipitate is pounded in an agate mortar, and preparations are made. It is sufficient to heat to 70° C. with frequent shaking.

c. Van Ketel's Method.—In a 100 c.c. flask provided with a wide neck are mixed 10 c.c. of sterile water,

6 c.c. liquid carbolic acid, and 10–15 c.c. sputum. This mixture is thoroughly shaken up and water added till the volume reaches 100 c.c. It is then shaken again and either allowed to deposit in a conical glass, or centrifugalized. Preparations before being stained should be washed in equal parts alcohol and ether.

d. Spengler's Method.—The sputum is mixed with an equal part of lukewarm water made alkaline by soda solution. It is then shaken up with 0·1–1·0 gram pancreatin powder and placed in the incubator at 37° C. Either at once, or after 2–3 hours, a crystal of carbolic of 0·2–1·0 gram's weight is added. When a sediment has formed, it is examined; the supernatant fluid being poured off. Should there be too much sediment, add further alkalinized water and pancreatin. Do not allow it to digest too long, and keep the reaction always alkaline.

e. Sachs-Müke Method.—Add small quantities of hydrogen peroxide repeatedly with constant stirring of the sputum, which becomes liquefied by the brisk evolution of gas. The bacilli can be found in the foam or sediment which falls after adding alcohol.

f. Loeffler's Method.—The sputum is boiled up with an equal amount of 50 % antiformin. To 10 c.c. of the mixture in a bottle with patent stopper are added 1·5 c.c. of a mixture of 10 c.c. chloroform and 90 c.c. alcohol. Centrifuge 15 minutes. Remove the layer which lies above the chloroform, transfer to a slide, blot, add a drop of egg white (containing $\frac{1}{2}$ % carbolic acid) and rub between two slides. After fixing stain with carbol-fuchsin, decolorize with absolute alcohol

containing 3 % HCl, wash in water and counter-stain with 0·1 % aqueous malachite green (chemically pure).

g. Ellermann and Erlandsen's Method.—10–15 c.c. sputum are kept at 37° C. for 24 hours in a corked measure with half the volume of 0·6 % NaOH. Decant, centrifuge the residue, decant. Treat the residue with four times its volume of 0·25% NaOH, stir thoroughly, boil, centrifuge and make preparations from the residue.

C. The Animal Experiment.—Though slower than *A* or *B*, this is the surest of all methods for the object in view. After subcutaneous (preferable when the material is rich in other bacteria) or intraperitoneal inoculation with tubercular material, guinea-pigs die generally in from 4–8 weeks with numerous tubercular nodules in the liver, lungs, and spleen, and with caseation of the lymphatic glands. In the nodules, which should always be examined microscopically to avoid confusion with other similar diseases, are numerous tubercle bacilli. For cultivation, one squeezes a nodule between two sterile slides and sows many pieces of it, each at least as large as a pin's head, on solidified blood serum, or on egg medium (p. 97). Growth becomes visible only after 8–14 days.

The animal experiment differentiates the tubercle bacillus from bacilli possessing similar acid-fast properties, viz. leprosy and smegma bacilli (the latter occur in urine—see below), which are not pathogenic to animals. In butter, milk, excrement, and on certain plants, also in the human body though rarely (e.g. in gangrene of the lung), there occur bacilli which stain in the manner of tubercle bacilli and

have similar pathogenic effect upon guinea-pigs, but which are distinguished from true tubercle by culture (e.g. they grow quickly on all culture media, and also at the room temperature).

If a guinea-pig inoculated with tubercle is killed 2–3 weeks after infection, one finds as a rule well-marked caseation of the lymphatic glands near the point of inoculation, and in its organs clearly developed nodules containing tubercle bacilli. In order to accelerate diagnosis, therefore, one may in case of necessity kill the animal without waiting for it to succumb to the disease.

Tubercle bacilli (Bovine type) obtained from cattle (Perlsucht) differ from the human type in their plumper form, growth on the surface of fluid media in the form of a delicate, hardly folded skin which does not reach the walls of the vessel, and also by their greater infectivity when inoculated into rabbits and cattle. In these animals they produce not only a localized reaction but also generalized tuberculosis. Similarly tubercle bacilli from the tuberculosis of birds and cold-blooded animals may be recognized by their special characters.

3. The Smegma Bacillus.

Smegma bacilli occur in preputial and vulvar secretion, and in urine; though the latter portions of urine withdrawn by catheter after previous cleansing of the external genitalia are mostly free of them. They also occur in the anal fold, in the secretion of the external auditory meatus, and occasionally in

other parts of the body. They are shorter and finer than tubercle bacilli, and they frequently lie upon the epithelium. On the other hand, tubercle bacilli in renal tuberculosis usually lie in clumps together and free from cells.

An attempt should be made to cultivate them on blood serum, or on egg medium (p. 97).

Staining properties. Smegma bacilli are acid-resistant in the manner of tubercle and leprosy. For their differentiation from tubercle, the animal experiment is available, and also the following staining methods:—

a. Honsell's Method.

1. Boil the preparation in carbol-fuchsin for two minutes.

2. Wash in water. Dry.

3. Treat in a mixture of absolute alcohol 97 parts, and HCl 4 parts, for ten minutes.

4. Wash in water.

5. Counter-stain in saturated alcoholic solution of methylene blue and aq., equal parts. Only tubercle bacilli retain the stain, the smegma bacilli parting with it.

b. Bunge and Trautenroth's Method.

1. Fix the cover-glass preparation in absolute alcohol for at least three hours.

2. Place in 3 % chromic acid for fifteen minutes, then wash.

3. Stain in boiling carbol-fuchsin for two minutes.

4. Treat with 5 % H_2SO_4 for 2–3 minutes.

5. Counter-stain with concentrated alcoholic solution of methylene blue for five minutes.

Only tubercle bacilli are stained red.

c. Pappenheim's Method.

1. Stain in boiling carbol-fuchsin for two minutes.

2. Allow the stain to drain off without washing.

3. Stain and counter-stain the preparation by dipping it 3–5 times in the following solution, which is allowed to slowly drain off after each immersion—dissolve one part of corallin in absolute alcohol 100 parts, add methylene blue to saturation, and glycerin 20 parts.

4. Rapidly wash in water. Dry, mount in balsam. Smegma bacilli blue, tubercle red.

4. The Leprosy Bacillus.

Cultivation of the leprosy bacillus has not yet succeeded with certainty. The attempt may be made to incubate a piece of leprous nodule with the addition of 0·8 % salt solution.

Staining is to be carried out according to the same methods described for tubercle bacilli. Leprosy bacilli are present in leprous tissue in larger numbers than tubercle bacilli in tubercular tissue, and are more easy to stain than tubercle bacilli. According to Baumgarten, differentiation between the two may be effected as follows: --

1. Stain in dilute alcoholic fuchsin (5-6 drops saturated alcoholic solution of fuchsin to a watch-glassful of water) for 6–7 minutes.

2. Decolorize for a quarter of a minute in alcohol 10 parts and HNO_3 1 part.

3. Wash in water. In case of sections, one then passes them through alcohol and cedar oil.

By this brief staining process leprosy bacilli are stained, but tubercle bacilli are not. But, in addition, the animal experiment is necessary for their differentiation; leprosy bacilli being non-pathogenic for animals.

In recent and suspected cases the nasal mucus should be examined in addition to the local lesions. Augmentation methods with antiformin should be tried as for tubercle (see Uhlenhuth, *Lepra*, vol. ix, sec. 2).

5. The Glanders Bacillus.

The greatest care should be exercised owing to the risk of infection.

B. mallei may be cultivated upon blood serum, agar, and potato at the body temperature. On potato it forms a reddish-brown membrane.

Pure cultures are best obtained by means of the animal body from pus of animals or men affected with glanders. After subcutaneous or intraperitoneal injection with the pus, field-mice succumb in 5–8 days, guinea-pigs in about 14 days. In the interior of the glanders nodules the bacillus is found present in pure culture. It is to be noted that swelling of the testicles, which, when the glanders bacillus is present, follows about two days after injection of the suspected material into the abdominal cavity of male guinea-pigs (Straus's method), is not absolutely pathognomonic of glanders since other bacilli present in disease processes simulating glanders can produce this effect. In all cases therefore make microscopical examination and cultures (see Kleine, *Zeit. f. Hyg.*, Bd. xlii, p. 183).

As regards method of diagnosis with killed bacilli for agglutination tests with the blood serum, see Ficker, *Hyg. Rdsch.* 1905, p. 649.

Staining characters. The glanders bacillus is negative to Gram. It may be stained with Loeffler's methylene blue (p. 67) or by Nicolle's Method (p. 70 *e* and *f*).

Special staining methods.

a. Loeffler's Method.

For film preparations.

1. Stain in either Loeffler's methylene blue (p. 67), or aniline water gentian violet, + 0·01 % caustic potash, equal parts, for five minutes.

2. Wash quickly in a 1 % solution of acetic acid tinted to about the colour of yellow Rhine wine by addition of tropaeolin OO (name of the dye) in watery solution.

3. Rapidly wash in aq. dest.

For sections.

1. Immerse the sections for a few minutes in 0·01 % caustic potash.

2. Stain as in above for films, for thirty minutes or longer.

3. Quickly wash in aq. dest. 10 + 2 drops concentrated sulphurous acid and 1 drop of 5% oxalic acid.

4. Decolorize in absolute alcohol. Then pass through cedar oil, &c.

b. Stain according to Kühne's carbol methylene blue method (p. 68), and for counter-staining add a little of either auranin or safranin dissolved in aniline oil to the oil of turpentine used for clearing.

c. Double staining according to Unna.

1. Dry the section on a slide and stain with Kühne's methylene blue (p. 68) for ten minutes.

2. Wash in water.

3. Stain for fifteen minutes in equal parts saturated watery solution of tannin and 1% watery acid fuchsin. (N.B.—Acid fuchsin is a special stain.)

4. Dehydrate in alcohol. Clear in oil of bergamot. Bacilli and nuclei blue, tissue reddish.

6. The Streptobacillus of Ulcus Molle.

Cultivate, if possible, on a mixture of blood and agar (p. 178, human blood is best). Cultures do not always succeed. The colonies lift off without breaking up. Long chains are found in the condensation fluid.

Cultures perish in a few days.

Smear preparations may be stained according to the usual methods. The bacillus is negative to Gram—if decolorization is carefully performed. Well suited for its demonstration are Nicolle's tannin method (p. 70) or Unna's method as follows:—

1. Stain with polychromatic methylene blue (to be obtained from Grübler, Leipzig) for two minutes.

2. Wash in water.

3. Remove the section with a spatula and dry it with blotting-paper.

4. Differentiate for 1–2 minutes in glycerine ether (from Grübler, Leipzig) a few drops to a capsule of water.

5. Wash in water carefully.

6. Dry on the spatula with blotting-paper.

7. Absolute alcohol. Bergamot oil. Balsam.

7. The Diphtheria Bacillus.

Cultures are to be obtained at temperatures above 20° C.—quickest at the body temperature. Growth occurs best on Loeffler's blood serum (p. 25) prepared from the sheep. On agar, glycerine agar, and the special media described below development is less profuse and the form of bacilli less typical.

The alkaline methylene blue of Loeffler (p. 67) is particularly well suited for staining the diphtheria bacillus. The bacillus stains by Gram's method (do not decolorize too vigorously), and is non-motile.

Diphtheria Diagnosis.

As regards removal of material from the throat for examination see p. 183. Surface cultures are made on Loeffler's serum solidified in tubes, or in Petri dishes (p. 25). N.B.—From each sample or batch of serum before it is used for diphtheria diagnosis, a few tubes must be tested as regards their nutrient capacity by inoculating them with pure cultures of diphtheria bacilli. Incubate at blood-heat (in case of necessity in an inner waistcoat pocket). Instead of Loeffler's blood serum the following media may be used.

1. *Glycerine Agar.* On this medium the colonies of B. d. remain small and are therefore less easily detected. Moreover, confusion between B. d. and pseudo-diphtheria bacilli is possible in consequence of similarity of form.

2. *Serum-Agar of Tochtermann.*

2 % watery solution of agar and 1 % peptone, 1·5 % NaCl and 0·3–0·5 % glucose are filtered and mixed with

lamb's or sheep's serum—which need not be sterile —either in equal parts or in ratio of serum 1 : agar 2. The medium is boiled for $\frac{1}{4}$–$\frac{1}{2}$ hour, filtered, filled into tubes and sterilized. It should not be boiled for longer than 1–$1\frac{1}{2}$ hours or its nutrient value is impaired. The colonies of B. d. are large and easily recognizable, but the bacilli are very similar to pseudo-diphtheria bacilli.

Loeffler's serum is much to be preferred to the above three media because the diphtheria bacillus grows on it quickly, abundantly, and in characteristic forms. The diphtheria bacillus is distinguishable from the Hofmann-Loeffler (throat) pseudo-diphtheria bacillus by its shape, and from the xerosis bacillus (the eye pseudo-dip. bacillus) by the same characteristic, but especially by the size of its colonies, which are far larger than those of xerosis.

Examination of the cultures has a prospect of success in from six hours after they have been sown with the material and placed in the incubator. Smear preparations are made from the larger parts of the surface and from the condensation water, and in case of plates, impression preparations are made. The preparations are stained with Loeffler's methylene blue. Diphtheria bacilli are recognizable by their characteristic morphology—stained and unstained parts of the bacilli alternate with one another; clubbed forms, and spindles are present. When no diphtheria bacilli are found in several preparations, frequent repetition of the examination may be made up to forty-eight hours after sowing. After 12–16 hours the colonies of B. d. generally become

typical and easily recognized (hemispherical, yellowish-white, moist) and preparations are then made from any such suspicious colonies. Further, Neisser's stain may be recommended (*Hyg. Rdsch.* 1903, No. 214).

1. Stain for about one second or longer in a mixture of 2 parts of the following solution A to 1 part B.

Solution A. Methylene blue powder 1.
Absolute alcohol 20.
Glacial acetic acid 50.
Aq. dest. 1,000.

Solution B. Crystal violet (Höchst) 1.
Absolute alcohol 10.
Aq. dest. 300.

2. Wash in water and at once.

3. Counter-stain in Cresoidin (cresoidin 1 part dissolved in warm water 300, and filtered) for about three seconds.

4. Wash in water.

The stain may be applied to smear preparations from serum cultures of from 14–20 hours' growth. The diphtheria bacilli show either at one pole or at both, and occasionally in the middle as well, a blue granule in the brown stained body of the bacillus, whereas in other bacilli of the diphtheria group these granules are not exhibited in the same time. The method is not absolutely trustworthy because sometimes, though not often, pseudo-diphtheria bacilli (xerosis and such-like) exhibit granules in the manner of B. d.; further B. d. itself sometimes shows granules at a later stage, and exceptionally not at all.

Loeffler recommends the following granule stain (*D. m. W.* 1907, No. 5) :—

1. Stain for 10 seconds in the following solution without warming :—

Borax 2·5%, methylene blue 1% in water, 40 c.c.
Unna's polychrome methylene blue (Grübler) 10 c.c.
0·05% aqueous Bromeosin extra A. G. (Höchst) 50 c.c.

2. Decolorize in the following solution :—

Saturated aqueous solution of tropaeolin OO 5 c.c.
Acetic acid 0·5 c.c.
Distilled water 100 c.c.

The bodies of the bacilli are stained pale blue, the polar granules dark blue.

For other similar staining methods see *Centrlbl. Bakt.*, Abt. I, Or.-Bd. xxxviii, p. 359.

When doubt arises whether the bacilli present are diphtheria or pseudo-diphtheria, therapeutically the case should be treated as diphtheria, antitoxin should be injected (if this has not already been done), and the suspicious bacilli present must be isolated in pure culture. If isolated colonies suitable for this purpose are not present in the original culture, a trace of the material is taken from a spot in which microscopical examination has proved the bacilli to be numerous and this trace is distributed in a tube of sterile broth, and from that a series of surface cultures made on serum tubes. Isolate from the latter as soon as possible. Often the differential diagnosis between diphtheria and pseudo-diphtheria is effected by the second series of serum cultures on account of the large number of colonies in the original culture, and the

consequent large number of bacilli in preparations therefrom. Should doubt still exist there follows:—

a. The Animal Experiment. A comparatively large platinum loopful of a pure culture on serum of 1–2 days' growth at 37° C., or from 0·2–1·0 c.c. of a pure culture in broth of the same age, is introduced subcutaneously under the chest of a guinea-pig of about 250 grams weight. If one is dealing with virulent B. d., the animal is ill with marked infiltration at the site of inoculation after one day, and dies as a rule after two days (organs sterile, suprarenals hyperaemic, large serous pleural exudation, and always in the first place infiltration, often haemorrhagic, at site of inoculation). When injected with the same dose of culture mixed with a sufficient quantity of diphtheria antitoxin (0·2 c.c. of a 200 fold or stronger serum) the animal remains alive, and the site of inoculation is entirely, or almost entirely, free of reaction. Pseudo-diphtheria bacilli, if injected in the same dose, produce at the utmost (and that very rarely) a minimum of oedema at site of inoculation, and do not kill; and if a reaction takes place at all it also occurs if diphtheria antitoxin is simultaneously administered.

b. Further reactions available for differential diagnosis.

Inoculate tubes each containing 10 c.c. of slightly alkaline sterile meat extract (p. 13) which must be recently made from fresh meat. The diphtheria bacillus forms acid (in 24–48 hours at 37° C. equal to about 0·35–1·0 of deci-normal H_2SO_4 with phenolphthalein as indicator) whereas the Hofmann-Loeffler

pseudo-diphtheria bacillus produces alkali (equal to about 0·2–0·4 of deci-normal NaOH).

Certain other pseudo-diphtheria bacilli occur on the skin, in wounds, pus, and in the conjunctiva, urethra, and vagina. Hine (*Journal of Pathology*, 1913) finds that in addition to glucose, saccharose and dextrin are useful for distinguishing these bacilli from B. d. which produces acid in dextrin as well as in glucose, but no acid in saccharose.

Toxin of the diphtheria bacillus.

This is present in the culture fluid, and may be prepared as follows :—

The diphtheria bacillus is cultivated at 37° C. in flasks of broth with a flat surface. It is advisable to prepare the broth from old meat, and to add powdered chalk to it. After 1–4 weeks filter free of bacterial bodies (p. 12). To the filtrate, which contains the toxin, $\frac{1}{2}\%$ carbolic is added for preservative purposes. One designates as *Normal Toxin* a toxin of which doses of 0·01 c.c., when subcutaneously injected into guinea-pigs of 250 grams weight, kill each of them in four or, at the outside, five days.

Immunization (see p. 209).

Diphtheria antitoxic serum is obtained from highly immunized animals. It is preserved by addition of 0.5% carbolic acid. One designates as *Normal Antitoxin* a serum of which 0·01 c.c. is sufficient to neutralize in the body of the guinea-pig a tenfold multiple of the minimum lethal dose of toxin. A tenfold normal serum is a serum of which 0·001 c.c. suffices, &c. For methods of testing see Ehrlich, *Klin. Jahrbuch*, Bd. vi.

8. The Influenza Bacillus.

Cultures only succeed at temperatures above 30° C., and only on agar either smeared, or better mixed, with blood. Enough blood should be added to redden the agar. Pigeon's blood is best, and can be obtained by puncturing a vessel on the inner side of the wing previously rendered sterile.

As medium also broth may be used with addition of ½–1% blood, in which case the medium should be frozen and then thawed again to liberate the haemoglobin. If practicable, all blood media should be incubated for twenty-four hours at 37° C. to prove sterility before use. The influenza bacilli are non-motile and very small. Monkeys alone can be infected by them.

For isolation of the influenza bacillus bronchial sputum is used which has first been freed of adhering mouth bacteria by washing it in several changes of sterile water. In a fatal case of influenzal pneumonia, juice from broncho-pneumonic patches of the lung can be mixed with 1–2 c.c. of broth till a uniform slightly turbid emulsion is produced. In consequence of this dilution, the number of transferred influenza bacilli is reduced so that isolated colonies can be obtained; and further, the haemoglobin contained in the material is so much diluted that growth will not take place on media to which blood has not been added. Platinum loopfuls of the dilution, therefore, can be sown on ordinary agar, glycerine agar, and blood agar. After twenty-four hours' incubation, one sees on the surface of the blood medium very small dew-drop-like colonies

of influenza bacilli, whereas no such colonies develop on the ordinary media. Colonies of B. i. in the neighbourhood of colonies of Staphylococcus aureus can assume large proportions. Bacilli morphologically and biologically very similar to influenza bacilli occur in the sputum in whooping cough and in certain types of conjunctivitis (e.g. Koch-Weeks bacillus).

For staining, one can employ Loeffler's methylene blue, or, better, carbol-fuchsin diluted with water till it has a pale red tint (p. 68). In the latter case the stain is allowed to act for a good many minutes. The influenza bacillus is negative to Gram. Sections can be stained by Pfeiffer's method (p. 69).

N.B.—Not all the cases called at the present day influenza are really influenza.

9. The Typhoid Bacillus

(including Paratyphoid bacilli).

B. typhosus can be cultivated on all the ordinary culture media. Growth takes place at the room temperature, and on slightly acid media. Pure cultures are most readily obtained from the spleen of the typhoid cadaver.

B. ty. stains with all the usual aniline dyes. It is negative to Gram's stain. Preparations made from cultures should be well fixed on the cover-glass, as the bacilli easily become detached. When examining sections made from the human spleen (differentiate very carefully because B. ty. is easily decolorized) a low power is used in the first place, and search is

made for masses of the bacilli which, if the stain is methylene blue, appear sky-blue, or in the case of carbol or aniline fuchsin bright red, or in case of thionin shining violet. (In order to obtain the bacilli in large masses the freshly removed spleen may be wrapped in a cloth moistened with sublimate to prevent decomposition, and placed in the incubator for twenty-four hours at 37° C.; under which circumstances the typhoid bacilli multiply greatly. After incubation the spleen is hardened.)

B. typhosus forms no spores.

Differential Diagnosis between B. typhosus and bacilli simulating it (*B. coli, B. faecalis alkaligenes, B. dysenteriae, and paratyphoid bacilli*).

1. B. typhosus exhibits pronounced motility (like a nest of ants). It possesses peritrichal flagella which are numerous, long, easily detached, and are readily demonstrable by Loeffler's stain to which 1% NaOH has been added (for details see p. 83).

2. B. ty. forms in gelatine plates deep colonies that are pale grey or yellowish in colour, and are round, oval, or shaped like a whetstone. The surface colonies have a delicate grey tint, and veil-like furrowing: only after several days do they become brown. No liquefaction is produced.

3. B. ty. forms on potato a scarcely perceptible layer of growth.

N.B.—Cultures of typhoid and of bacilli resembling it which one desires to compare should be made either on pieces of the same potato, or at different spots on the same slice.

4. B. ty. grows in milk, but without coagulating it. (Cultures should be kept for several days at 37° C.)

5. B. ty. produces in litmus whey (p. 49) not more than 3 % of deci-normal acid (the whey remains clear).

6. B. ty. does not produce gas in glucose (see p. 47). (Stab cultures in glucose agar are specially to be recommended for this test, or else culture in glucose broth in fermentation tubes. Optimum at 37° C.)

7. B. ty. forms no indol (for method of testing see p. 50).

8. B. ty. gives the proteinochrome reaction (p. 51).

9. B. ty. does not change the colour of neutral red agar (add to nutrient agar containing either 0·3 or 0·5 or 0·75 % agar, and 0·3 or 0·5 % glucose, 1% of a cold saturated watery solution of neutral red in water and sterilize in the steamer). Inseminate either by stabbing well-filled tubes or by inoculating the medium when fluid. In place of agar, 10% gelatine may be used and kept at 37° C.; B. coli and similar micro-organisms produce a green fluorescence which later becomes bleached; and some of them form in addition gas.

10. B. ty. leaves unchanged a dilute litmus solution + 1 % nutrose, 0·5 % NaCl, and 1% lactose, during cultivation therein for twenty-four hours at 37° C. This is a distinction from B. coli, which causes the medium to become red, coagulates it, and produces gas. If the medium contains 1 % glucose in place of the lactose, B. ty. produces redness and coagulation within twenty-four hours at 37° C. This is a distinction from the

dysentery bacillus which does not produce redness within twenty-four hours, and only produces coagulation later.

Certain micro-organisms can only be distinguished from B. ty. by application of all the above ten tests. For instance, B. faecalis alkaligenes is only with certainty differentiated by test 5 and in similar manner by test 10 (it forms alkali); B. dysenteriae by test 1 (absence of motility), by test 10, and by the further actions referred to under B. dysenteriae (p. 138). Moreover, the **Paratyphoid** bacilli which give rise to diseases clinically resembling typhoid, of which two forms have been differentiated, are in many respects similar to typhoid. The rare **Paratyphoid A** forms circular colonies without markings in 2, the commoner **Paratyphoid B** similar colonies but thick white and when young iridescent, and later surrounded by a slimy wall, stains potato brown, slowly decolorizes milk with production of alkali, renders litmus whey after 8–10 days' alkaline. Both form gas and fluorescence in test 9, and produce coagulation in litmus glucose nutrose solution. But although no micro-organisms other than typhoid have been found to answer all the ten tests in the manner of that organism, the surest means of diagnosis is by—

11. **The Serum Reaction**, which has two forms, viz. (*a*) bacteriolysis in the animal body and (*b*) agglutination.

(*a*) **Bacteriolysis in the animal body** (the so-called Pfeiffer's Reaction).

Principle. B. ty., when mixed with the serum of an animal highly immunized against it, and injected

into the peritoneal cavity of a guinea-pig, is quickly dissolved (it is caused to undergo obvious dissolution); but if in place of the immune serum, serum from a normal animal is used, this dissolution does not take place. The bacilli that simulate typhoid are affected neither by the immune nor by the normal serum. (The reaction only holds when there is a definite quantitative ratio between serum and culture, and when the strain of B. ty. used is of proved virulence.)

The following mode of conducting the test is recommended:—

a. Exact determination of the potency of the immune serum (Titre estimation).

This is to be ascertained by means of a virulent culture of B. ty. of which about $\frac{1}{10}$ loop = 0·2 mg. (for method of dose determination see p. 207) suspended in 1 c.c. of broth kills a guinea-pig of about 250 grams weight in the course of about twenty-four hours by intraperitoneal injection, whereupon a fall of temperature and vigorous multiplication of the bacilli takes place. The serum is diluted with nutrient broth in such manner that 1 c.c. of the mixture contains 0·01, 0·005, 0·001, or even less of the serum. A loopful of a twenty hours' agar culture of the before-mentioned virulent typhoid strain (e. g. the tenfold fatal dose) is suspended in each c.c. of the serum-broth mixture, and each c.c. is then injected into the peritoneal cavity of a different guinea-pig. (The skin of the abdomen should be incised and the injection made through a blunt cannula passed through the muscular wall.) At different intervals (30, 60, and 120 minutes) tests are made of the peritoneal contents by means of

capillary pipettes. The peritoneal fluid ascends to a short distance in these pipettes, and a drop of it is examined in a hanging drop preparation. (The contents of the capillary tube may be expelled on a cover-glass by closing the upper extremity with the finger and warming the pipette in the hand.) If after two hours, at the latest, most of the bacilli have disappeared, or have disintegrated into granules, and only a few isolated bacilli are seen and these non-motile, then proof is obtained that the dose of serum introduced into the animal protects against the action of the bacilli. The animal in that case survives without marked fall of temperature. The smallest dose of serum that just suffices for this result is the titre of the serum. The serum employed for the reaction should, whenever possible, have a titre of 0·001 or under (e.g. 0·0001). For method of obtaining immune serum see p. 209.

b. Serum of a normal animal of the same species as that yielding immune serum. (This has similar protecting effect to the immune serum according to experiment only in quantities of tenths of a cubic centimetre.)

c. An agar culture of about twenty hours' growth at 37° C. of the bacillus of test.

d. Two guinea-pigs each of about 250 grams, two syringes, pipettes, sterile empty test-tubes, and broth tubes.

Guinea-pig *A* receives an intraperitoneal injection consisting of a suspension of one loopful (=2 mg.) of an agar culture of the bacillus of test in 1 c.c. of a mixture of broth and immune serum corresponding

to ten times the titre of the serum (perhaps also another stronger one, but never exceeding 0·02 c.c. —for details see *Possibility 3*).

Guinea-pig *B* receives the same dose of culture in a mixture of 0·95 c.c. broth and 0·05 c.c. normal serum.

After 30, 60, and 120 minutes examine the peritoneal contents (withdrawn by capillary pipettes) and keep the animal under observation. There can take place: —

Possibility 1. In guinea-pig *A* the bacilli disappear quickly, and the animal survives. In guinea-pig *B* the bacilli do not disappear but multiply, and the animal dies with fall of temperature. Inference—the culture is genuine typhoid, since the anti-typhoid serum has altered it, while the normal serum used in larger quantity has not done so.

Possibility 2. In guinea-pigs *A* and *B* the bacilli do not disappear and the animals survive. Inference—the culture is of too feeble a virulence to carry out the test. (N.B.—Increase of the dose of culture to over one loopful is not permissible.) In this case, perhaps, the experiment may be made of increasing the virulence by passage through the animal body, e.g. (1) an animal succumbs if two loopfuls are injected intraperitoneally, and an agar culture is made from its peritoneal contents. (2) Another animal dies on intraperitoneal injection of one loopful of this culture, and an agar culture is made as before. (3) A third animal dies on intraperitoneal injection of $\frac{1}{4}$ loopful —now the reaction should be tested with one loopful of culture and serum as above described.

Possibility 2 of course only comes into consideration in respect of genuine cultures of B. ty., and then only when such cultures have been kept in culture for a considerable period. B. ty. freshly isolated from the body is always of good virulence.

Possibility 3. The bacilli disappear in neither guinea-pig *A* nor *B*, and both animals die. Inference —the culture is no typhoid culture. Cultures of B. ty. of such virulence that even a tenfold multiple of the titre of the immune serum is not able to protect guinea-pigs against infection with one loopful of them do not occur. If such existed, then indeed it is obvious that this inference would not be justified. But it follows from the same consideration that if less powerful sera than mentioned above are employed, this inference must not be drawn without further proof. Assuming that one has serum of a titre of 0·01 c.c., and one uses 0·02 c.c. of it (more is not permissible or the action of normal serum may come into play), then the serum dose used protects against twenty times the fatal dose of culture (titre against tenfold also against the double quantity, i.e. twentyfold). But if the culture to be tested has a M.L.D. of $\frac{1}{50}$ loop, and one injects an animal with a dose of serum and one loopful of culture, then it is clear that the quantity of serum injected is not sufficient to protect against this amount of culture (fiftyfold M.L.D.):—and even though the dissolution of the bacilli is not complete, the culture may, nevertheless, be typhoid. Therefore either employ only a powerful serum, or when only a low-grade serum is available, test the virulence of the culture of test by injecting intra-

peritoneally a series of guinea-pigs with doses of from $\frac{1}{10}$–$\frac{1}{100}$ of a loopful of it. Then inject such a multiple of the culture (mixed with serum as described above) that the quantity of serum admissible (0·02 c.c.), provided the culture is typhoid, with certainty suffices for protection. In that case, when *Possibility 3* occurs, the inference drawn above is justified.

(*b*) **Agglutination** (Gruber, Durham, R. Pfeiffer).

Principle. Whereas serum from an animal immunized against typhoid renders B. ty. non-motile and, when in high dilution, causes it to aggregate into clumps, serum from a normal animal produces such effect only when in strong concentration. Typhoid serum affects the bacilli simulating B. ty. at the utmost somewhat more markedly than normal serum does.

N.B.—Typhoid bacilli recently cultivated from the body are sometimes agglutinated by typhoid serum only after repeated culture on artificial media.

Mode recommended for carrying out the tests.

a. Immune serum of known potency. Diminishing quantities of typhoid serum are added to emulsions of virulent B. ty. in 0·8% NaCl solution in small test-tubes (0·5–0·8 cm. wide, 6–8 cm. long, with conical base) and by means of hanging drop preparations at 37° C. one observes first of all by what dilution of the serum immobility and agglutination of B. ty. is produced within at the latest two hours. The smallest quantity that suffices for this purpose is the titre of the serum for the hanging drop (or microscopic) method. Secondly, one determines by what addition

of serum to the tubes there is produced within at the latest two hours, precipitation of flocculi to the foot of the tube visible with the naked eye or with the hand lens in reflected light. The smallest quantity that suffices for this purpose is the titre of the serum for the tube (or macroscopic) method (titre determined by hanging drop confirmed). The serum should be efficient in a dilution of at least 1 : 1000 of 0·8 % NaCl solution in the tube experiment. Sera of higher potency are, according to some authorities, not to be recommended since they also act to a certain extent on typhoid-simulating bacilli. On the whole, errors are comparatively infrequent ; for it is very seldom that the immune serum used agglutinates typhoid-simulating bacilli in the manner of genuine typhoid.

b. Normal serum from an animal of the same species as that yielding the immune serum. Examine the titre of this normal serum in the same manner as described under *a*. The normal serum must be of very much lower potency than the immune serum (e.g. titre of normal serum 1 : 20, of immune serum 1 : 1000).

c. An agar culture of twenty hours' growth at 37° C. of the bacillus to be tested.

One prepares different dilutions of the immune serum with 0·8 % NaCl solution, e.g. 1 : 200, 1 : 300, 1 : 500, 1 : 1000. The lowest dilution of immune serum should be at least ten times as dilute as the titre of normal serum (e.g. titre of normal serum 1 : 20, strongest concentration of immune serum used 1 : 200). One fills 1 c.c. of each dilution into one of the little narrow tubes (see under *a*), labels the tube care-

fully, distributes in it a small loopful (2 mgr.) of the culture of test, and very carefully grinds up the bacterial material with the fluid, holding the tube in a sloped position. Now make from each tube a hanging drop preparation, and place these and the tubes in the incubator at 37° C. Further test as a control similar admixture of the same culture with normal serum diluted with broth and make up to 1 c.c., conducting the test in such manner that the serum is present to half its titre strength (e.g. when the titre is 1 : 20, use in the control 1 : 40). Examine every ten and twenty minutes up to two hours.

There can take place :—

Possibility 1. In the case of the immune serum, the bacilli are clumped either in all the tests, or only in those tests in which the largest quantity of serum is present ; negative results are obtained with the normal serum. The culture is typhoid. (This inference, however, is open to doubt when the difference between the titre for B. ty. and the dilution of the serum that agglutinates the bacillus of test is very great, e. g. titre 1 : 5000 and agglutination of the test organism only 1 : 50, negative 1 : 100. In such a case it is best to make Pfeiffer's test.)

Possibility 2. The bacilli clump in both series (very rare). In this case the culture may be typhoid of diminished virulence (possibly there are indications of clumping with quantities of the immune serum below the titre). The virulence of the culture should be examined, and perhaps the reaction re-tested after the virulence has been increased (see p. 121 above).

Possibility 3. The bacilli clump in neither of the two sera. If one is employing strong immune serum, the inference may be made that the culture is not typhoid.

Still, it sometimes happens that typhoid bacilli freshly cultivated from the body are not agglutinated until they have been subcultured many times on artificial media. The test must therefore be repeated.

For method of obtaining immune serum see p. 209.

Serum from a typhoid convalescent is not applicable for differential diagnosis, since it is generally too feeble for this purpose, and besides frequently agglutinates bacilli that simulate typhoid (see p. 135 (*c*)).

Bacteriological Diagnosis of Typhoid Fever.

The surest method is the demonstration of B. typhosus in the blood or evacuations—less reliable is the test of the agglutinating capacity of the serum. When circumstances permit, it is best to begin with No. 1 or 2 of the methods described below, and to follow up, if possible, with Nos. 3 and 4, and even also 5. Micro-organisms of the typhoid type recovered from the body are always to be tested by all the criteria described above (p. 116, 1–10, 11 (*b*) and perhaps also 11 (*a*)), since maladies resembling typhoid occur which are produced not by B. typhosus, but by bacilli simulating that micro-organism.

1. Examination of the Rose Spots.

Cleanse the skin by gently rubbing with alcohol and ether. Make a superficial incision into the rose spot

with a scalpel; and at once with its point scrape up some of the tissue juice before blood makes its appearance, and sow in broth. Place on the wound two drops of broth in order to reduce the bactericidal power of the exuding blood. Sow in broth the thus diluted blood. Should the bacillus grow, it must be identified according to the directions given above. Always examine several rose spots, and fresh ones only. The method is successful in about three-quarters of all cases.

2. Examination of the Blood in Typhoid.

Withdraw 5–20 c.c. of blood from the median vein by means of a syringe (see p. 179, *B*). Sow each ten drops of it in 20 c.c. of broth, or mix the blood with warm—about 49° C.—agar in the proportion of three of agar and one of blood, and make plates. Typhoid and paratyphoid colonies appear dark green. The method is successful in about 90 % of the cases already in the first week of the disease. Sowing in agar is to be preferred, because thereby the number of bacilli present is established; and in case of contamination, isolated colonies are present from which pure cultures can be made. Primary culture by incubation of a mixture of 2·5 c.c. blood with 5 c.c. of boiled ox gall (12–24 hours at 37° C.) is regarded by some as useful. This admixture, in which the blood remains uncoagulated, is to be recommended also when sending material for investigation by post. See Conradi, *D.m.W.* 1906, No. 2; Kayser, *Centrlbl. f. Bakt.*, Abt. I, Or.-Bd. xlii, p. 185.

3. Examination of the faeces for B. typhosus.

a. By the usual plate method in which neutral, or not quite neutral, gelatine is employed as a medium, B. ty. may not rarely be demonstrated in the stool, especially after the second week of the disease. This result is achieved by subculturing from the plates a large number of the colonies resembling typhoid, testing these cultures for gas formation (p. 117), and further studying by the methods described above, such of these cultures as do not produce gas from sugar.

b. Far better is the following litmus-nutrose-agar method of Drigalski and Conradi (*Zeit. f. Hyg.*, Bd. 39).

1·5 kilogram of minced horseflesh is extracted with 2 litres of water for twenty-four hours without heat. Boil for one hour the meat extract strained off from this, filter it, and add 20 grams of peptone sic. (Witte), 20 grams nutrose, and 10 grams NaCl. Boil for one hour, filter, add 60–70 grams of the finest agar, and boil for either three hours in the steamer or one hour in the autoclave. Make feebly alkaline to litmus-paper, filter, and boil for half an hour. To this agar, when it has cooled somewhat, is added litmus-lactose-solution warmed to 40-50° C. (litmus solution from Kahlbaum, Berlin,—260 c.c. is boiled for ten minutes, 30 grams of chemically pure lactose are then added to it, and the whole is boiled for fifteen minutes, not longer). Shake well, and restore the slight alkaline reaction if it has disappeared. Add to the mixture 4 c.c. of hot sterile solution of 10% anhydrous soda in water, and further, 20 c.c. of freshly-prepared solution of 0·1 gram crystal violet (Höchst)

in 100 c.c. of warm sterile distilled water. Pour part of the mixture into large Petri dishes 15 cm. in diameter (layer of medium at least 2 mm. thick), and keep ready for use. The other part should be filled into 200 c.c. flasks (not large flasks, because these take too long in melting). Make surface cultures by distributing the culture material with a glass rod bent at a right angle. Thin stools should be distributed on several plates in succession, both directly and after dilution with 10–20 times the quantity of 0·85% sterile salt solution. Solid stools should be distributed after dilution to thin consistency by the above-mentioned salt solution. After inoculation, take the covers off, and allow the plates to stand for at least half an hour inverted; then replace the covers and incubate at 37° C. Examine after 14–24 hours: typhoid colonies are 1–3 mm. in diameter, blue, glassy, not double contoured, like drops of dew: and paratyphoid colonies are similar to them. Coli colonies are 2–6 mm. across, bright red, opaque. The subcultured colonies must be further tested according to the methods of differentiation described above (p. 117). Identification is effected most speedily by agglutination on the cover-glass (p. 123).

[The following more convenient way of preparing the Drigalski and Conradi medium may be recommended:—

A.

Meat extract	1 litre.
Peptone (Witte's) . . .	10 grams.
Nutrose	10 grams.
Chloride of Sodium . . .	5 grams.

Directions:—Mix the nutrose in a beaker with a little of the meat extract and make into a paste, gradually adding a little more meat extract from time to time till the mixture is quite fluid and free from lumps; now pour the mixture into a flask containing remainder of the meat extract, add peptone, salt, and about 4 c.c. of 10 % sodium carbonate solution. Bring slowly to the boil over a Bunsen burner with rose flame. Boil for 1 hour, shaking occasionally to prevent the nutrose from burning. Allow to stand for a short time, or over-night if possible, pour off the supernatant fluid and make up to 1 litre with meat extract.

(Nutrose dissolves much more readily in alkaline solutions.)

B.

Agar fibre . . . . 30 grams.

Prepare agar in same way as previously described (p. 18) by acetic acid method. When drained, add the nutrose peptone broth and heat in autoclave to 115° C. for 45 minutes, or in steamer for 90 minutes, filter as for agar. When filtered, heat in steamer for 30 minutes.

C.

Kubel and Tiemann's litmus solution . 130 c.c.
(Prepared by Merck.)

Place in flask, boil for 10 minutes, add 15 grams of *chemically pure lactose* (Grübler) and boil for a further ten minutes.

Add to the filtered nutrose-peptone-agar, and mix thoroughly; add 2 c.c. of 10 % sterile sodium carbonate solution, and further add 10 c.c. of a freshly prepared sterile solution of 0·1 % crystal violet.

Put about 20 c.c. in tubes and steam in steamer for 30 minutes on two successive days.]

c. The Fuchsin Medium of Endo is also good (*Centrlbl. f. Bakt.*, Abt. I, Or.-Bd. xxxv, p. 109).

1 litre of 3 % nutrient agar (p. 17) is neutralized, and then made alkaline with 10 c.c. of 10 % soda solution. Ten grams of chemically pure lactose are added, and 5 c.c. of saturated alcoholic filtered fuchsin solution. Then is added 25 c.c. of freshly-prepared 10 % solution of sodium sulphite. The medium, which in a heated condition is rose-coloured, should be preserved in the dark after it has cooled and the original colour faded. Inoculate in Petri dishes, as in case of (*b*). Typhoid and paratyphoid colonies are colourless; coli colonies are stained intensely red. Do not subculture till after 20–22 hours. Endo-tablets, which when added to neutral agar yield Endo's medium, can be obtained from Merck of Darmstadt.

d. Combination of method (*b*) with caffein media, according to Roth, Ficker, and Hoffmann (*Arch. f. Hyg.*, Bd. 49, pp. 199, 229).

To 100 c.c. of beef extract are added 6 % peptone (Witte), 0·5 % NaCl, and 38·64 % of normal NaOH—the quantity of alkali that is needed to cause the medium to react red to phenolphthalein (the mode of testing and calculation is described on p. 20, No. 4), then sterilize for ten minutes in the steamer. When sterile, add 105 c.c. of 1·2 % solution of caffein and 1·4 c.c. of 0·1 % solution of crystal violet (both dissolved in cold sterile distilled water). Thin stools are added directly (0·8–0·9 c.c.) to the mixture.

Thicker fluid stools and solid stools are crushed up with 1–2 parts of 1·2 % solution of caffein in sterile Petri dishes, filtered through sterile wool, and 0·8–0·9 c.c. of the filtrate sown. After incubation for thirteen hours at 37° C., cultivate on litmus-nutrose-agar (p. 128).

e. Loeffler's Malachite green-safranin-blue agar (*D. m. W.* 1909, No. 30). To a litre of 3 % neutral nutrient agar are added 5 c.c. normal NaOH and (at conclusion of sterilization) 100 c.c. of 10 % nutrose solution; the mixture is preserved in flasks of Jena glass and cleared by sedimentation. For use 100 c.c. of the agar are liquefied and cooled to about 45° C. and to it are added 3 c.c. filtered ox gall (sterilized by boiling), 1 c.c. of 0·2 % aqueous 'Pure Safranin' (Grübler), 3 c.c. of 1 % aqueous 'Pure-blue double concentrated' (Höchster Farbwerke), and 3–4 c.c. of 0·2 % aqueous malachite green crystals (Höchst). The whole is thoroughly mixed, poured into plates and inoculated. Typhoid colonies are blue, transparent, low pyramids with uneven surface and metallic sheen after rather more than twenty-four hours. Paratyphoid B is very similar; Paratyphoid A colonies are round, glassy, and bluish, those of Gaertner's bacillus round and red, and those of B. coli red or reddish.

Lentz and Tietz (*Klin. Jahrb.*, 1905, p. 495) employ the malachite green medium for obtaining multiplication of typhoid and paratyphoid bacilli. The stool is crushed up in an equal quantity of 0·8 % NaCl solution, and sown on the surface of a malachite green plate (malachite green No. 1, Höchst 1 : 6000 of agar). After twenty-four hours' incubation, at 37° C., examine

for typhoid and paratyphoid colonies. If none is found, suspend the surface growth of the plate in about 8–10 c.c. of broth, and inoculate from the uppermost layer of this broth, which has been allowed to stand for some time in the plate, which is sloped.

4. The Serum reaction of Widal.

Principle. The blood serum of a typhoid patient possesses as a rule already after the first eight days of the disease the power of agglutinating the typhoid bacillus. Serum of patients suffering from diseases other than typhoid produces agglutination only when in stronger concentration. (But see p. 135 under (*a*).) For method of obtaining sample of blood see p. 178. Note.—Various strains of typhoid differ considerably in their facility of agglutination, a strain which has been proved satisfactory by being tested with an immune serum should therefore be selected.

Method of conducting the agglutination test (see also the directions published in the *K. G. A.* 1903, No. 36).

By means of a 1 c.c. pipette, graduated to $\frac{1}{100}$ c.c., a measured quantity of the serum is diluted fifty times with sterile 0·8 % NaCl solution. When this initial dilution bulks under 2 c.c., the test of agglutination is confined to cover-glass observations; but if otherwise, from 0·5–1 c.c. of it is placed in test-tubes (for form and size of these tubes see p. 123.

Microscopic test.

Drops of the 1 : 50 dilution are placed on cover-glasses, and on some more cover-glasses drops from a

1 : 100 dilution are placed. By means of a platinum needle traces of growth from a forty-eight hours' agar culture of typhoid and of paratyphoid *A* and *B* respectively are distributed in these drops; enough being added in each case to produce definite turbidity to the eye. The needles are then flamed, each drop is rubbed down so that its bacteria form an even suspension, and the cover-glasses mounted as hanging drop preparations and examined.

Macroscopic test.

To 1 part of the 1 : 50 dilution of the serum an equal volume of 0·8 % salt solution is added, thus forming a dilution of 1 : 100. Both dilutions are then placed in test-tubes and examined with regard to their capacity of agglutinating respectively B. ty. and B. paraty. *A* and *B*. To this end, in each 1 c.c. of serum dilution, one normal loopful (=2 mg.) of culture is carefully suspended by rubbing it down at the edge of the test-tube. The tubes are then allowed to stand for three hours at 37° C., or from evening till next morning at the room temperature.

Examination of the reaction.

The agglutination that takes place in the tubes should always be controlled by the microscope, especially when the serum contains blood corpuscles. The test made with the 1 : 50 dilution is only of value as an indication. If there are in every field numerous clumps of 6–7 bacilli in a preparation in which originally only isolated bacilli were to be found, and in which even now some of the bacilli remain well isolated, the reaction may be considered positive. If

in the tube test 1 : 50 is positive, but not 1 : 100, the case is suspicious, and the test should be repeated in a few days' time with a fresh sample of the blood serum. If the test with paratyphoid alone is positive, the case is probably one of that disease ; but it should first be proved to be so by cultivating the paratyphoid bacillus from the patient. In positive cases it is recommended that further dilutions of the serum should be tested in order that the limit of the agglutinating capacity of the serum for typhoid and paratyphoid bacilli respectively may be established.

The Widal serum reaction has numerous sources of error, more especially the following :—

(*a*) The serum of persons who have had typhoid months or years before, but who at the time being may have another disease, can agglutinate B. ty. The serum of icterus cases also often agglutinates B. ty. in high dilution.

(*b*) The agglutinating capacity of the serum may not develop in the usual time ; and sometimes it does not appear at all. When a negative result as regards agglutination occurs in a case in which on clinical grounds there is suspicion of typhoid infection, it is recommended that the test should be repeated after an interval of a few days.

(*c*) In typhoid infection the blood serum possesses a capacity of agglutinating paratyphoid bacilli, and vice versa. In doubtful cases, therefore, it is advisable to test agglutination of high dilutions such as 1 : 100, 1 : 200, 1 : 500, &c., against both B. ty. and B. paraty. The bacillus causing the disease is agglutinated by a

higher dilution than the others which are responding to what is known as the ' group reaction '.

As support for the clinical diagnosis, Widal's reaction possesses great value when correctly interpreted. Therapeutically, and from the point of view of sanitary administration, both typhoid and paratyphoid are treated similarly.

One should never confine oneself to the statement that Widal's reaction is positive, but should also state the kind of test applied (tube or cover-glass), the dilution in which the serum is effective, the time within which agglutination takes place, and the intensity of the reaction (complete agglutination, &c.).

A preparation of the typhoid bacillus and also of paratyphoid for Widal's reaction, free of living organisms, is to be obtained from P. Merck, Darmstadt (Ficker's typhoid diagnosticum *B. Klin. W.* 1903, No. 425) and is applicable for the test-tube experiment and convenient in practice. An alternative preparation may be made by adding to a one day's broth culture of typhoid 1% of 40 % formalin, by keeping the culture thus treated for two days at 37° C., and by then pouring off the fluid from the precipitate and storing the former in the ice chest. This suspension keeps well and agglutinates tardily, though always easily.

B. ty. occurs in urine in about one quarter of all cases of typhoid, but usually not before the third week. It is best detected in this material by the methods described on pp. 128–33 *b*, *c*, and *e*.

After recovery from typhoid, bacilli may be excreted in the stools and urine for months and even years.

Therefore in cases of doubtful illness a careful history should be taken.

The Isolation of B. typhosus from Water.

Mode of procedure recommended:—cultures should be made from the water as directed under General Water Examination (p. 220), but on the media described on p. 128. (Perhaps also sediment of the centrifuged water might be spread over the surface of these media.) Use a large number of plates. Subculture suspicious colonies and test these for gas formation, and in other respects (see p. 128, 3 *a*). Special methods of examination are numerous. Compare for instance the literature given in Müller's paper, *Zschr. f. Hyg.*, Bd. 51, p. 1; but their value has still to be established.

10. The Dysentery Bacillus.

A. The type of Shiga and Kruse.

In form and growth B. dysenteriae presents an extraordinary similarity to B. typhosus, but is plumper and non-motile. It may be cultivated from the faeces (from flakes of mucus) on litmus-nutrose-agar (p. 128, without the crystal violet—grows like B. ty.). The serum of dysentery cases shows at first tardy agglutinating capacity for the bacillus, and agglutination by a 1 : 50 dilution of the serum is proof of dysentery.

Chief characters.

1. Non-motile (but well pronounced molecular movement). Flagella not found present (distinction from B. typhosus).

2. No gas formed in glucose-agar (for details see p. 117 under 6).

3. No indol-formation (details of test p. 117 under 7).

4. No coagulation in milk.

5. Acid is formed in litmus-whey as in the case of typhoid (see p. 117 under 5).

6. Grows on litmus-nutrose-agar (pp. 128–31) in the manner of B. typhosus. The medium should be prepared without crystal violet.

7. Growth in litmus-nutrose-agar made with mannite (prepared as described on p. 128, but instead of lactose the same quantity of mannite is used) or in litmus-mannite-agar (nutrient agar with addition of 10 % litmus solution and 2 % mannite). B. dysenteriae in stab cultures leaves the upper layer unchanged, but decolorizes the lower parts of the medium. B. typhosus, B. coli, and most of the other organisms simulating B. dysenteriae stain the medium red, a few stain it blue, and some form gas.

8. Agglutination by the serum of an animal immunized against B. dysenteriae (for method of immunization see p. 209). Test as in case of B. typhosus, p. 123. Immunization of animals is difficult on account of toxicity—sheep and goats, and especially the horse and ass, are best.

For further distinctions from B. typhosus compare p. 117, No. 10, but the difference is one of time, and so uncertain.

B. Flexner's type.

Behaves as type *A*, but reddens litmus-mannite-agar (see above) in twenty-four hours, grows on litmus-nutrose-agar with addition of crystal violet (see under

6 above), and forms indol. In addition, it agglutinates only with specific serum, and not with the serum which acts on type *A* (see above), despite the fact that there is a certain affinity in their agglutination capacities (compare 'Group-reaction', page 135, paragraph (*c*), for details as to mode of determining the degree of this).

Agglutination with the serum of a patient is not definite proof of infection even in a dilution of 1 : 100 or higher, since normal serum sometimes agglutinates to that degree. The immunization of rabbits and other animals readily succeeds.

In addition to these two types there are also strains differing from them in various properties which are allied to the Flexner type and together with it are called 'Pseudo-dysentery bacilli'. They are distinguished by their growth on litmus-sugar media and by their agglutination reactions with immune sera prepared with their aid. Further, in dysentery stools there are frequently found, in addition to dysentery bacilli, other allied non-motile bacilli which in part coagulate milk.

11. Bacillus (or Bacterium) Coli.

The typical representatives of this group are fairly motile, possess from 4–12 lateral flagella, form on the surface of gelatine a good film-like layer of growth, and in the depth, round brownish colonies without liquefaction.

On potato they form pronounced greyish-brown growth ; strongly ferment glucose ; form indol ; show no proteinochrome ; clot milk; produce in litmus-whey

strong and lasting acid reaction. Varieties occur which lack one or more of these normal characters: no varieties, however, liquefy gelatine. For differentiation from B. typhosus and B. dysenteriae see pp. 116, 137. Negative to Gram's stain. Pathogenic for guinea-pigs by intraperitoneal injection (peritonitis, septicaemia); sometimes also for these animals and for mice by subcutaneous injection (suppuration, septicaemia). Agglutination with specific sera analogous to that described p. 123; though an immune serum does not act on all strains, but only on that against which it has been prepared and perhaps a few others. *Eijkmann's Test*, see page 224.

[B. coli has been used for a number of years past as a means of detecting excremental pollution of water. The test, however, was without definite quantitative basis until Houston showed (*L.G.B.* 1902–3, *ib.* 1903–4) that typical B. coli, possessed of reactions for which he has introduced the abbreviated term FLAGINAC (FL = fluorescence in neutral red media; AG = acid and gas in lactose; IN = indol; and AC = acid and clot in litmus milk), is a bacterial test whereby 0·000,000,01 gram and less of human excrement may be detected. Owing, therefore, to the fact that it forms the most delicate and trustworthy index at present available for recognizing the presence of excremental pollution, and (what is still more important) for determining the degree to which such pollution obtains, B. coli, especially typical or Flaginac B. coli, provides the most valuable test which Science has yet been able to furnish for gauging the purity of a water-supply. For details

as to procedure recommended in examining water, milk, &c., by the B. coli test see pp. 221, 223 et seq.]

12. The Vibrio of Asiatic Cholera.

Cultures of V. cholerae asiaticae are obtainable on the ordinary culture media. A somewhat alkaline reaction is favourable. The growth on gelatine is particularly characteristic—in plates the colonies resemble small collections of flakes of glass, and in stab culture there is clear air-bubble-like liquefaction of the surface. On agar, bluish transparent colonies are formed. Growth occurs abundantly in peptone water (p. 23), and on potato only when the latter has been boiled in soda solution (1%) or in salt solution (p. 28). The vibrio has a terminal flagellum. It exhibits darting motility—like a swarm of gnats. No formation of spores; cultures give the nitroso-indol reaction (p. 50). No phosphorescence is exhibited.

Staining is best effected with fuchsin solution. The vibrio fails to stain with Gram's stain.

Differential Diagnosis between Cholera and Vibrios simulating it.

This can often be effected by means of the gelatine plate culture, and by observing the nitroso-indol reaction and phosphorescence; but it is only effected with absolute certainty by employing the serum reaction in the form of bacteriolysis in the peritoneal cavity of the guinea-pig, or the agglutination test (for details see pp. 146, 147).

Bacteriological Diagnosis in a case of Cholera. (See directions for prevention of cholera issued by the Imperial Board, Berlin, Rich. Schoetz, 1905.)

Examination is made either of a stool, or of the contents of an intestinal loop from the middle or lower part of the ileum. The material is spread out in a large glass double capsule, and flakes of mucus are fished out for examination. In the case of solid evacuations, soften with sterile water in order to find the flakes.

I. Method of Examination.

1. *Microscopical examination.*

a. Smear preparations (made if possible from flakes of mucus). Stain with dilute carbol-fuchsin (1 : 9 aq.).

b. Examine in hanging drop preparations made with peptone water (p. 23) both at once, and after being kept for half an hour at 37° C., and in each instance make both fresh and stained preparations.

2. *Gelatine plates.* The quantity to be sown is one loopful (if possible from a mucous flake), and also a dilution to the third loop. Make two series of cultures of three plates each. After keeping them for eighteen hours at 22° C., examine with a low power, prepare impression preparations, perhaps also smear preparations; and make pure cultures.

The gelatine should be made of meat extract (p. 13) and 1% peptone, $\frac{1}{2}$% salt, 10% gelatine, and after this mixture has been rendered neutral to litmus (see p. 14) 3% of a 10% solution of crystallized soda is added. (For alkali-albumen gelatine see p. 149.)

3. *Agar plates.*

Either spread out a loopful of the material on the surface of three agar plates in succession, or distribute a loopful of the material in 5 c.c. of peptone water, and inoculate plates each with a single loopful of this dilution. After 12–18 hours at 37° C. examine as directed under 2, *supra*.

The agar should consist of meat extract, with 1% peptone, ½% salt, 3% agar; and, as in the case of the gelatine, it should be slightly alkaline. Before inoculation, the plates should have their covers taken off and should be kept inverted in the incubator at 37° C. for half an hour.

4. *Augmentation by means of peptone solution* (see p. 23).

(*a*) Several tubes containing 10 c.c. of the peptone solution are inoculated each with a loopful of the material. After keeping these tubes for 6–12 hours at 37° C. examine the surface of the fluid microscopically, being careful not to shake the tube. From each of the tubes most suspected of containing cholera vibrios, three further peptone-water tubes are then inoculated, each subculture receiving one loopful, and a series of agar and gelatine plates are likewise prepared from each. Before inoculation, the peptone-water tubes should be warmed to 37° C. in the incubator.

(*b*) Sow about 1 c.c. of stool in a flask containing 50 c.c. of the peptone solution, incubate and examine as under (*a*).

5. *Preparation of pure cultures.* These are best obtained from the agar plates. Make gelatine stab, or agar streak cultures.

6. *Proving of the pure cultures.* These are tested (*a*) by determining their capacity of agglutination, and (*b*) by Pfeiffer's test.

II. Procedure.

1. In primary cases (i.e. when no case of cholera has yet been observed in the locality) all of the methods should be used, and in the following order:—(*a*) inoculate six peptone-water tubes, (*b*) make microscopical preparations, (*c*) prepare in each instance a series of gelatine and agar plates, (*d*) examine microscopically, (*e*) prepare pure cultures, (*f*) test these according to the methods described above.

2. In secondary cases (i.e. when the presence of cholera in the place has already been established) one should proceed as just described, but only three tubes of peptone-water need be inoculated; only one series of gelatine and agar plates; and perhaps instead of agar plates sloped tubes may be used. As regards (*f*), it is sufficient to test agglutination.

3. In suspected cases (i.e. in cases of diarrhoea) and in convalescents, the microscopic examination is omitted if the stools do not resemble cholera. Instead of the peptone tubes, use a peptone flask (see above 4 (*b*)), and from this after 6–12 hours' incubation at 37° C. prepare in each case a series of gelatine and agar plates. Test suspicious colonies as directed above in I. 6.

4. *Water examination.* At least 1 litre of the

water is added to 10% concentrated peptone-water solution (p. 23), and thoroughly mixed. Each 100 c.c. of the mixture is then poured into a flask, and after 8–18 hours at 37° C. a hanging drop and a stained preparation are made from the surface of the fluid of each flask. From those flasks that contain micro-organisms most nearly resembling cholera vibrios, peptone-water tubes and gelatine and agar plates are made and further examined as described above. The pure cultures should be tested according to both the methods referred to under I. 6 (*a*) and (*b*).

III. Interpretation of the Results.

As regards II.—1. The diagnosis of cholera is only to be regarded as reliable when all the methods give a positive result, and of particular importance are the two tests referred to in I. 6. If the microscopical examination shows vibrios in the characteristic arrangement (in collections like a school of fish, in stained preparations), nearly in pure culture, and the colonies on the gelatine plates are typical, then a provisional diagnosis of cholera may be made; but before making a final diagnosis the result of the whole of the examination should be awaited. If the result of the agglutination test of suspicious colonies in the hanging drop ('indication' test of agglutination) is not unequivocal, then one must proceed to quantitative estimation of agglutination as soon as a pure culture has been obtained (see p. 147).

As regards II.—2. The diagnosis of cholera depends upon a positive result in the microscopical examination, upon the growth on gelatine and agar plates

being characteristic, and upon positive agglutination in the hanging drop test.

As regards II.—3. In suspected cases cholera may be considered excluded when several separate examinations of the faeces in one day carried out as described under II. 2 fail to show the presence of the cholera vibrio. Convalescents are to be regarded as no longer capable of spreading infection when three examinations made at intervals of one day yield negative results.

As regards II.—4. Water vibrios are to be regarded as cholera vibrios when their agglutination capacity corresponds in degree to that yielded by the cholera vibrio, and Pfeiffer's test is positive.

IV. Detection of Cases of Cholera that have recovered from the Disease.

Such cases—suspected of having had cholera—can be detected by examination of their blood serum. Blood may be withdrawn by cupping, whereby at least 1 c.c. of serum can be obtained. This serum is diluted either with 0·8% salt solution or with broth, and examined with regard to its capacity for agglutinating a fresh cholera culture, and also in Pfeiffer's test (see p. 147).

The Agglutination Test (suitable serum can be obtained from the Institute of Infectious Diseases, Berlin).

a. *Examination in the hanging drop with low magnification.*

Two different dilutions of the specific serum, i. e. the lowest limit of activity against a test-culture and

another five times as strong, should be made with 0·8% salt solution, and the vibrio must give obvious clumping at 37° C. within twenty minutes at the latest. As a control make parallel test, using ten times as strong concentration of normal serum from an animal of the same species as that furnishing the specific serum: here agglutination must be negative. It should be borne in mind that agar cultures under fifteen hours old of some strains of vibrio can only be emulsified with difficulty, and thus may simulate agglutination.

b. *Quantitative determination of the agglutination capacity.*

Dilute the specific serum with absolutely clear 0·8% NaCl solution (most conveniently passed twice through thick filter-paper), making dilutions of 1 : 50, 1 : 100, 1 : 500, 1 : 1000, 1 : 2000. In each dilution, which bulks to 1 c.c. and is placed in a tube, emulsify one loopful of agar culture and distribute it equally throughout the medium by shaking. After keeping the emulsions for one hour at 37° C. examine them in reflected light with a hand lens. Only undoubted clumping is to be recorded as positive. Agglutination should set in in regular steps until the limit is attained. In each test make a control experiment, examining (1) normal serum of the same kind of animal as that yielding the specific serum, but in ten times higher concentration + the culture, (2) the 0·8% NaCl solution + the culture, and (3) a trustworthy cholera culture of the same age as the culture of test + similar dilutions of the specific serum.

Pfeiffer's Test (suitable serum can be obtained from the Institute of Infectious Diseases, Berlin. It should

possess a titre of at least 0·0002 mgr., i.e. this amount of serum should suffice to cause granular degeneration and dissolution within one hour of one loopful (= 2 mgr.) of an eighteen hours' cholera culture of stable virulence, when this amount of culture is suspended in 1 c.c. of broth and injected into the peritoneal cavity of a guinea-pig).

Four guinea-pigs, *A–D*, each of 200 grams weight, are inoculated. *A–C* each receive intraperitoneally one loopful of an eighteen hours' culture at 37° C. of the vibrio of test suspended in 1 c.c. of broth and mixed in the case of *A* with five times the titre dose (e.g. 0·001) of the specific serum; in the case of *B* with the tenfold titre dose (e.g. 0·002) of specific serum; and in the case of *C* (control animal) with the fivefold multiple of the titre dose of normal serum from an animal of the same species as that furnishing the specific serum. Guinea-pig *D* receives only one quarter loop of the culture under examination, and serves as a test of its intraperitoneal virulence.

Hanging drop preparations are made from the peritoneal fluid after twenty and after sixty minutes (as in the expt. described on p. 119 *a*). If the culture is cholera, the vibrios are dissolved after at the latest sixty minutes in *A* and *B*, whereas in *C* and *D* numerous lively well-preserved vibrios are present.

As regards IV (cholera cases that have recovered), the following method is practised.

The human serum is diluted with 20, 100, and 500 parts of broth. To each of these dilutions, which are in all cases made up to a bulk of 1 c.c., is added one loopful (2 mgr.) of an eighteen hours' agar culture of

virulent cholera, and the emulsions are then severally injected into the peritoneal cavity of guinea-pigs. If on examination after 20–60 minutes bacteriolysis is found to have taken place, the case furnishing the serum is to be regarded as having undergone an attack of cholera. A control guinea-pig of 200 grams receives intraperitoneally no serum, but one quarter loopful of the culture suspended in 1 c.c. of broth.

The addition of alkaline blood to agar aids the development of the cholera vibrio and inhibits that of the usual intestinal organisms. For its preparation equal parts of defibrinated ox blood and normal potash solution are mixed and sterilized for half an hour in the steamer. Thirty parts of the mixture are added to seventy of neutral agar and poured into plates which are dried somewhat by being left in the incubator. After 24 hours (owing to the formation of ammonia which inhibits growth) the plates are inoculated by streaking (Dieudonné, *Centr. f. Bakt.* I, Or. 50, p. 107). The following somewhat similar medium can be inoculated in plates after standing for about an hour at room temperature:—Rub up 5 gr. Haemoglobin (Merck) in a mortar, dissolve in 15 c.c. normal NaOH and 15 c.c. distilled water in a flask with gentle warming. Sterilize in steam for an hour and add 15 c.c. of the mixture to 85 c.c. neutral agar (Esch, *D. m. W.* 1910, p. 559).

(The cholera vibrio forms a very characteristic growth on alkali albumen gelatine (Deycke). Alkali albumen can be obtained from E. Merck, Darmstadt (100 grams=11 Mk), or may be prepared as follows: 1 kilogram of the finely-minced fat-free flesh of a calf

is extracted for forty-eight hours at 37° C. with 1200 c.c. of 3% KOH, and then for several hours at 60–70° C. Filter off the juice and add to it diluted HCl until no more precipitate forms. Filter off this precipitate and dissolve it in the steamer in sufficient concentrated soda solution to make a fairly alkaline solution of the precipitate. Evaporate off the fluid and dry the remainder at 100° C. to a powder. 2–3 grams of this powder, or of the albumen which is purchased, is mixed with 8 grams of peptone, 1 gram NaCl, 10–15 grams gelatine, and 100 c.c. of aq. com. to form a nutrient gelatine. Neutralize with HCl, and render alkaline with $\frac{2}{3}$ % crystallized soda.)

13. The Plague Bacillus.

(See directions for prevention of plague issued by the Imperial Board, Berlin, Rich. Schötz, 1905.)

The preservation of living cultures of B. pestis, as also the scientific study of the same with animal experiments, is confined in Germany to certain specially constructed laboratories, and in practice cultural examinations are only allowed for the purpose of determining the diagnosis in suspected cases.

The material examined in cases suspected of plague consists chiefly of bubo juice or pus (obtained by syringe or incision), blood, sputum, and secretion from the throat. Staining is carried out with the usual stains. For polar staining dry preparations are fixed in absolute alcohol for twenty-five minutes (better still, immerse in alcohol for one minute

and then get rid of the alcohol by warming before staining: thin watery methylene blue is best). Cultivation may be effected on agar, blood serum, or gelatine (feebly alkaline; for impure material gelatine is best). In the first generation there is rather slow growth (forty-eight hours and more may elapse before obvious colonies develop). The bacillus is non-motile, and does not stain by Gram's method. On agar containing 3 % NaCl, spherical and yeast-like forms develop. In broth chains are formed. Sugar is not fermented. Gelatine is not liquefied. Pathogenic for rats, guinea-pigs, and other animals by all methods of infection: particularly characteristic is the cutaneous mode of infection by rubbing the organism into the shaved and scarified skin of the guinea-pig. The bacillus is agglutinated by the serum of immunized animals and of convalescents from plague.

14. The Tetanus Bacillus.

B. tetani grows only in the absence of oxygen. To obtain a pure culture from pus derived from a case of tetanus one inoculates broth with the material, passes hydrogen through the tube, and places it in the incubator at 37° C. After twenty-four hours, or more certainly after forty-eight hours, the bacilli have formed spores, of which fact one can convince oneself by making preparations. If the cultures are now heated for $\frac{3}{4}$–1 hour to 80° C. in a water bath, only the tetanus spores survive (provided that very resistant spores of other bacteria are not also present). By

anaerobic plate cultures one can now obtain isolated colonies from which pure cultures may be made.

Cultivation from earth, &c. In the first place mice or guinea-pigs may be infected subcutaneously. From these animals, dead after a few days with tetanic symptoms, cultures may be prepared from pus at the site of inoculation. The bacillus grows at the room temperature and liquefies gelatine.

The bacillus stains with the ordinary aniline dyes, and is positive to Gram's stain. The spores (drumsticks) may be stained by the methods described on p. 80. In the body, the bacilli are found only at the site of infection. They are motile with peritrichal flagella.

15. Bacillus Botulinus.

Motile by means of 4–8 flagella, possesses terminal spores and is Gram-positive. It is an obligatory anaerobe, grows best at 18-25° C., and liquefies gelatine. Even very small doses of a broth culture injected subcutaneously into animals quickly produce motor paralysis, dilatation of the pupil, &c., and finally death. The organism is found in decomposed meat, and does not multiply in the body.

16. Bacillus Pyocyaneus.

Growth takes place on all the ordinary culture media. The organism is motile and has one flagellum. Typical strains form dark-green pigment (pyocyanin),

which gradually becomes brown and is soluble in chloroform. Similar fluorescent bacilli lack this pigment. Pathogenic for rabbits and mice. Negative to Gram's stain. Liquefies gelatine.

17. Pyogenic Staphylococci and Streptococci.

Cultures are obtainable on the usual culture media, growth occurring best at blood heat. Staphylococcus aureus and albus liquefy gelatine, Streptococci and Tetragenus do not. All four are positive to Gram's stain. Streptococcus chains are best seen in fluid media (also in agar condensation water).

Blood examination in Septicaemia and Pyaemia.

Blood is withdrawn from the median vein by a syringe (see p. 179), and immediately sown in six tubes of melted agar of a temperature about 42° C., each tube receiving 2–3 c.c. of blood. Pour the tubes out into plates, invert the latter, and incubate them for 1-4 days at 37° C. Make also cultures in deep tubes (see p. 42) since there are also anaerobic pathogenic streptococci (Strep. pubidus). The following may be found:—

Streptococcus pyogenes seu erysipelatis. Small clear colonies with sharp margin. (Haemolysis.)

Streptococcus mitis seu viridans (seldom found). Very small greenish colonies, renders glucose agar turbid. Usually non-pathogenic for animals.

Streptococcus mucosus. Very delicate green; colonies are markedly slimy (seldom found, mostly in pneumonia). Highly pathogenic for animals.

Staphylococcus pyogenes. Luxuriant colonies with sharp margins.

Gonococci. Small opaque colonies.

Pneumococci. Small opaque colonies.

[It has recently been shown that both Streptococci and Staphylococci can readily be differentiated *inter se* by their biochemical reactions.]

Differentiation of Streptococci. (Gordon, *L.G.B.*, 1902-3, 1903-4; Houston, *ib.* 1903-4, 1904-5, also Report to L.C.C. on milk standards 1905; Gordon, Report on Ventilation of House of Commons 1906; Andrewes and Horder, *Lancet*, Sept. 1906.) The tests of value for differentiating streptococci are:—(1) the production of clot in litmus milk at 37° C.; (2) the production of green fluorescence in neutral red broth during incubation for 2 days anaerobically at 37° C.; (3) the production of an acid reaction when cultivated for 3 days aerobically at 37° C. in slightly alkaline Lemco fluid containing 1 % saccharose; (4) ditto, but lactose; (5) ditto, but raffinose; (6) ditto, but inulin; (7) ditto, but salicin; (8) ditto, but coniferin; (9) ditto, but mannite.

The Lemco medium (Houston) in which the substances used in tests 4-9 are dissolved consists of distilled water containing Lemco 1 %, peptone 1 %, sodium bicarbonate 0·1 %, and it is tinted with litmus.

Reactions of some of the chief types of Streptococcus (Andrewes and Horder, *Lancet*, Sept. 1906):—

No.	Name.	Milk Clot.	N. Red.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Coniferin.	Mannite.	Growth on Gelatine 20° C.	Morphology.	Pathogenicity for Mouse.
1	Streptococcus Pyogenes	±	−	+	+	−	−	±	−	±	+	Longus	+
2	,, Salivarius	+	±	+	+	±	−	−	−	−	±	Brevis	−
3	,, Anginosus	+	±	+	+	−	−	−	−	−	±	Longus	+
4	,, Faecalis	+	+	+	+	−	−	+	+	+	+	Brevis	−
5	,, Equinus	−	−	+	−	−	−	+	+	−	+	Brevis	−
6	Pneumococcus	±	−	+	+	+	±	−	−	−	−	Brevis	+

In view of some recent criticism of these tests, it may be stated that the chief points of distinction between the reactions of the three commonest types of streptococcus *as met with in the human body* are as follows :—

Type.	Neutral red.	Raffinose.	Mannite.
S. pyogenes	−	−	?
S. salivarius	+	?	−
S. faecalis	+	−	+

In addition to the above physiological differences, there are morphological and cultural differences between these three streptococci, e.g. salivarius and faecalis are both more oval than pyogenes, and while

faecalis and pyogenes grow readily at 20–22° C., salivarius frequently fails to develop at this temperature.

Differentiation of Staphylococci. (Gordon, *L.G.B.*, 1903-4; *ib.* 1904-5.)

The biochemical tests of value for differentiating Staphylococci consist of (1) clotting of milk; (2) liquefaction of gelatine; (3) production of green fluorescence in neutral red broth; (4) reduction of nitrate to nitrite; (5) production of an acid reaction in the Lemco medium containing maltose; (6) ditto, but lactose; (7) ditto, but glycerine; (8) ditto, but mannite. The neutral red test is applied in the same way as in the case of Streptococci; the other tests require to be applied for a week at 37° C. instead of only for 3 days as in case of the Streptococcus tests.

The reactions of some Staphylococci of human origin in these tests are as follows:—

Name.	Milk Cloth.	Gel. Liq.	N. Red.	Nitrite.	Maltose.	Lactose.	Glycerine.	Mannite.
Staphylococcus pyogenes aureus	+	+	±	+	+	+	+	+
Staphylococcus epidermidis albus	+	+	+	+	+	+	+	−
A staphylococcus sometimes found on skin	−	−	−	−	+	+	−	−
A staphylococcus found in scurf	−	−	−	+	−	−	−	+

[The mannite test, it will be observed, readily distinguishes the first two staphylococci in the above table.]

18. The Pneumococcus and Pneumobacillus.

Pure cultures are most readily obtained by subcutaneously injecting mice or rabbits with pneumonic sputum, and by sowing the blood of these animals—which perish in forty-eight hours from so-called sputum septicaemia—on agar or serum. From the saliva of many persons also the pneumococcus may be obtained in the same way. Growth occurs best at blood heat (colonies like little drops of dew). No growth takes place below 20° C. Cultures should be subcultured every 4–6 days, for they often tend to rapidly die out. In blood pneumococci can preserve their vitality for several months.

Fraenkel's pneumococcus stains with the ordinary stains, and is positive to Gram. Friedlander's bacillus, however, is negative to Gram. The latter forms at the ordinary temperature in gelatine stab a nail-shaped growth, and grows on agar in a slimy white glassy layer. This organism is larger than the pneumococcus, is shaped as a bacillus, and in mice on subcutaneous injection produces septicaemia. Capsules may be demonstrated round both the pneumococcus and the pneumobacillus in preparations made from the body. For methods of capsule staining see p. 78.

Pneumococci are differentiated from some of the pyogenic streptococci only by their 'candle-flame' shape, by their formation of capsules in the animal body, and by their tendency to form mostly diplococci and only very short chains in fluid media. For reactions of pneumococci in the differential tests for streptococcus see p. 155.

19. The Meningococcus.

(The Micrococcus of Epidemic Cerebrospinal Meningitis.)

Growth is only good at temperatures above 25° C. For its cultivation from the body (e.g. from purulent brain-exudation, cerebrospinal fluid, nasal secretion, or secretion from the posterior pharynx) Esch's blood agar is best (*Centr. f. Bakt.* I, Or. 52, p. 150; 60 c.c. nutrient agar, with 1% Witte's peptone, to which are added 20 c.c. sterile defibrinated sheep's blood, 10 c.c. ascites fluid, and 1 gr. maltose dissolved in broth); also acites agar, Loeffler's serum, or placenta agar are good. In broth the coccus produces turbidity and flocculi. The first generation shows feeble growth, but later, growth is more luxuriant. Cultures should be kept moist in the 37° C. incubator, for otherwise the coccus quickly dies. It must be subcultured daily when freshly isolated, but later every 5–7 days. The organism is Gram-negative and stains particularly well with Loeffler's method for gonococci, see p. 163. The shape is similar to that of the gonococcus, and the cocci are also mostly enclosed in cells. The coccus is feebly pathogenic (toxic) for animals (mice and guinea-pigs) on intra-peritoneal injection. As a rule the serum of the patients agglutinates the coccus (above 1 : 50 is characteristic). For identification of cultures of the coccus, agglutination with the serum of immunized animals is applicable.

M. catarrhalis and numerous other cocci that occur in the nose and pharynx are similar in form to the meningococcus, and also do not stain with Gram. They grow well, however, on ordinary agar, also on gelatine

(without liquefaction), and are to be distinguished by sugar and agglutination tests.

Such differentiation can be accomplished by litmus-sugar-media (Gordon, *B. M. J.* Aug. 1905 ; von Lingelsheim, *Klin. Jahrb.* xv, p. 410 ; Rothe, *Centr. f. Bakt.*, Or. 46, p. 645). To 10 % solutions of sugars in litmus solution (Kahlbaum) boiled for two minutes are added after cooling 0·5 c.c. normal soda solution per 10 c.c. Of the solutions so obtained 1·5 c.c. are added to 13·5 c.c. of a mixture of three parts of 3 % nutrient agar and 1 part ascites fluid. Plates are poured and stroke preparations are made. Meningococci colonies turn red with dextrose or maltose, gonococci with dextrose only, other cocci not even with dextrose but may do so with laevulose.

[Identification of the meningococcus in cultures from blood and from cerebrospinal fluid withdrawn by lumbar puncture offers no particular difficulty, because, when present in these fluids at all during life, the meningococcus is usually in pure culture. In the case of mucus from the upper respiratory passages, however, identification of the meningococcus is much more difficult, because Gram-negative cocci of various kinds are normally present in this secretion, and in point of fact commonly exceed 100,000 per c.c. of normal saliva (Gordon, Report to L.G.B. 1907). The characters of chief value for differentiating the meningococcus from these Gram-negative cocci of the normal throat are inability of the meningococcus to grow below 23–25° C. ; the agglutination test ; and its biochemical behaviour when cultivated in the presence of glucose, maltose, and saccharose respectively.

Gram-negative coccus	Glucose.	Maltose.	Saccharose.
Meningococcus .	+	+	–
Gonococcus . .	+	–	–
Catarrhalis . .	–	–	–
A common throat coccus . .	+	+	+

The following medium—nutrose ascitic agar, or more briefly 'nasgar'—is slightly modified from one described by Wassermann (p. 162), and is very useful for cultivation of the meningococcus.

A. Ascitic fluid 15 c.c., distilled water 35 c.c., nutrose 1 gram. Put in a flask, shake well, and slowly boil over a Bunsen burner with rose flame for 30 minutes, occasionally whirling flask to prevent burning. The nutrose prevents the ascitic fluid from coagulating.

B. Agar fibre 3 grams, peptone (Witte) 1 gram, sodium chloride 0·5 gram, meat extract to 100 c.c. Soften the agar fibre by the acetic acid method previously described.

Add 1 part A to 2 parts B, cool to 60° C., add the beaten whites of two eggs (for a quantity of about 1 litre). Put in a flask and heat in autoclave to 115° C. for 45 minutes, or in a steamer for 90 minutes. Filter through a moistened Chardin's filter-paper (32 cm.), placing the funnel in a warm water-jacket. Put in tubes and heat in the steamer for 30 minutes on 2 successive days.]

20. The Gonococcus.

Cultures on ordinary laboratory media are not successful—at all events not in the first generation, though sometimes apparent strains of the gonococcus grow slightly on them in later generations. Use may be made of the following media, on which at a temperature of 36° C. (preferably not higher) the gonococcus grows in small dewdrop-like colonies.

1. *Solidified human blood serum prepared according to Bumm.*

When in a delivery the cord has been ligatured as usual with a couple of ligatures, and severed between these ligatures, the placental end of it is disinfected and cut through above the proximal ligature. The blood that exudes is collected in a sterile vessel, and further dealt with in the same way as animals' blood (see p. 23). Should the placenta be already expelled, it may be subjected to manual pressure. On the medium, when solidified, surface cultures are made from the material containing gonococci. Growth is not luxuriant.

2. *Human blood serum and agar mixed according to Wertheim.*

Each of three sterile test-tubes receives 1 c.c. of fluid serum obtained as in 1, and all are then warmed to about 40° C. The first tube is inoculated with the gonorrhoeal material, from it the second is inoculated, and from that the third tube; 2 c.c. of melted agar of a temperature of about 40° C. are then poured in each tube, and the mixture poured out at once into plates, allowed to solidify and incubated.

It is better still to smear the material over the surface of plates already solidified. Colonies are larger than in the case of 1.

3. *Ascites agar according to Kiefer.*

Neutral meat extract agar, containing 3·5% agar, 5% peptone, 2% glycerine, and 0·5% NaCl, is melted, and when it has cooled to 50° C. is mixed with an equal quantity of ascites fluid and allowed to solidify either in Petri dishes or in sloped tubes. Surface cultures are made on this medium. If the ascites fluid is very alkaline, then one mixes with it unneutralized or strongly acidified agar solution so that the mixture is slightly alkaline.

4. *Blood-smeared agar according to Abel.*

One sows the material on nutrient agar previously smeared with a little blood derived from some portion of the human skin, which has been disinfected and then quite freed of the disinfectant by washing it with sterile water and a plug of sterile wool. The first generation does not always grow. Recommended for subcultures because it is simple to prepare.

5. *Nutrose medium according to Wassermann.*

Mix in a flask 15 c.c. of pig's blood, 30–40 c.c. of water, 2–3 c.c. of glycerine, 0·8 gram of nutrose, and while constantly shaking the mixture, boil for fifteen minutes. Repeat the boiling and the shaking on the following day for fifteen minutes. This fluid is kept ready, and when it is to be used it is heated to between 50–60° C., mixed with an equal quantity of 2 % peptone agar of the same temperature, poured into plates, and used for surface cultures. Especially recommended for subcultures.

To test whether the colonies that have developed really consist of gonococci one makes :—

a. Preparations, and stains both with the ordinary stains and with Gram's stain (in contradistinction to most of the other cocci, gonococci fail to stain with Gram).

b. Subculture on ordinary agar. If the micro-organism is the gonococcus there is no growth (this applies at any rate to freshly isolated cultures. See head of subsection).

c. Inoculation of animals (mice, rabbits—the gonococcus is only toxic in large doses).

d. Cultivation or litmus sugar media (see above under Meningococcus).

Staining of gonococci in preparations from pus, &c. (diplococci mostly in the pus cells) may be effected with the ordinary dyes, or if it is desired to counter-stain the tissue elements the following methods may be used :—

1. *The eosin and methylene blue stain*, described on p. 71. Gonococci and cell-nuclei blue, rest of the tissue red.

2. *According to Loeffler* (*D. m. W.* 1907, No. 5).

(1) Fix the film in equal parts of alcohol and ether.

(2) Stain with solution 1 (see p. 111), warming gently.

(3) Wash in water.

(4) Decolorize in the following mixture :—

Absolute alcohol 177 c.c.

0·1% aqueous Bromeosin B. extra (Höchst) 20 c.c.

Acetic acid 3 c.c.

(5) Wash in water.

Gonococci dark blue, nuclei pale blue, cells pale red.

3. *According to Giemsa* (see p. 172).

4. *According to A. Neisser.*

(1) Stain in warm saturated alcoholic solution of eosin for several minutes.

(2) Drain off the eosin with blotting-paper and at once.

(3) Pour on to it saturated alcoholic solution of methylene blue ($\frac{1}{4}$ minute).

(4) Wash in water.

Gonococci and cell-nuclei blue. Cell bodies red.

5. *According to Pappenheim.*

Stain for one minute in a solution of about 2 pen-knife-points of methylene green, about $\frac{1}{2}$ a knife-point of pyronin (Grübler, Leipzig), dissolved in 5 c.c. of aq. dest. (The solution should be blue-violet: find by experiment the best mixture.) Wash in water. Gonococci red, nuclei blue.

Secretions containing few gonococci (from old cases of gonorrhoea) are spread out on the slide (p. 60) and stained by Gram's method. Gonococci take the counter-stain. Be careful at each washing with water in this process until the preparation has been decolorized in alcohol.

21. Actinomyces.

Cultures are to be obtained on the ordinary culture media, especially on glycerine agar. Make many cultures, including anaerobic ones, since some strains grow only in absence of air. Growth of the first generation is usually slow but later becomes more

abundant. The growth is dry, and firmly adheres to the medium which the colonies, as it were, eat into; later the growth is chalk-white or yellow, and besprinkled with powder. Growth also occurs on gelatine at the room temperature, and both this medium and serum are (always ?) liquefied by it. (Clubs are not found in cultures, but in young cultures coccus and rod-like forms and in older ones threads.) Actinomyces nodules from pus may be crushed up in a sterilized agate mortar in order to sow them better.

Actinomyces can be stained by the ordinary staining methods, but in the case of sections the fungus is not so well demonstrated, and counter-stains are preferable as follows :—

a. Gram's method (see p. 73). Sections should be stained for twenty-four hours. (According to Loeffler, one should first place the preparation for a few minutes in 0·01% caustic potash.) Then for fifteen minutes in potassium iodide. Counter-stain with eosin or vesuvin.

b. Weigert's method (see p. 76).

c. Weigert's method with Orseille.

1. Stain in a dark-red solution of orseille in absolute alcohol 20, acetic acid 5, aq. dest. 40, for 1–24 hours.
2. Wash in water.
3. Stain in 1% watery solution of gentian violet.
4. Clear in cedar oil.

Cell-nuclei blue-violet; tissue orange; actinomyces nodules internally pale blue, externally ruby red, and in between often a colourless zone.

N.B.—In order to free the orseille of ammonia one leaves it exposed to the air for some time before use.

d. Israel's method.

1. Stain the section for several hours in a concentrated solution of orcein in water acidified with acetic acid.

2. Wash in water ; remove the section with a spatula and dry it with blotting-paper.

3. Place for a few seconds in absolute alcohol.

4. Quickly place the section on a slide ; dry it by first pressing on it with blotting-paper, and then allowing it to dry in the air.

5. Canada balsam.

If one wishes the fungus to be stained, but not the tissue, this is effected by allowing the alcohol to act for a longer time. The result of the stain is similar to that seen in *c.*

e. Bostrom's method.

1. Stain in aniline water gentian violet.

2. Transfer without washing to picrocarmine (as in Weigert's method, see p. 77).

3. Wash in water.

4. Decolorize in absolute alcohol till the sections are reddish-yellow.

5. Clear in cedar oil—Canada balsam.

Centre of nodules pale blue, clubs red, tissue yellowish-red.

22. Yeasts, and Thrush Fungus.

Cultures should be made on feebly acid media containing sugar. Particularly favourable is sterilized beer wort or grape juice, and either agar or tap-water gelatine without further addition. Isolation

to be effected by plate methods (p. 31), or by preparation of a single cell culture as follows:—Very greatly dilute in gelatine, prepare a hanging drop, examine with low magnification and mark the spots where the isolated cells lie by means of ink marks on upper side of cover-glass. Subculture from the colonies that develop in these spots.

Staining is successful with the usual aniline stains.

Staining of the spores can be effected by staining with boiling carbol-fuchsin, washing in 4% sulphuric acid, and contrast-staining the cell substance with methylene blue.

Staining of the Nuclei by Möller's method.

1. Place the preparation for at least two hours in 3-4% solution of sulphate of iron and ammonia.
2. Wash in water.
3. Stain for half an hour in saturated solution of haematoxylin in spring water.
4. Wash in water.
5. Differentiate in solution 1 for $\frac{1}{2}$–2 minutes, constantly controlling the process by the microscope.
6. Wash in water; dry in the air; Canada balsam.

Busse's method for demonstrating Yeasts in the tissues.

1. Stain in haematin for fifteen minutes.
2. Wash in water for five minutes.
3. Stain in Ziehl's carbol-fuchsin (p. 68) 1 : 20 aq. for $\frac{1}{2}$–24 hours.
4. Differentiate in 60% alcohol, absolute alcohol, cedar oil, &c.

Tissue nuclei blue, yeasts (not all) brilliant red.

23. Moulds and other Fungi.

For the cultivation of moulds one employs the usual culture media, especially acid media, bread pulp (p. 29), and the media described for cultivation of yeasts. Of the commonest moulds the pencillia as a rule grow only at the room temperature ; a few kinds of aspergillus and mucor, however, grow at blood heat, and are pathogenic for rabbits by intravenous injection.

In making a microscopical examination one, if possible, examines with a low power the upper surface of the mould as it rests upon the medium, and then examines a piece of it laid between coverslip and slide without addition of water. If it is desired to examine, in fluid, moulds that have grown in the air, water is not used, because this fails to moisten them properly, but one first wets the piece of mould on the slide with alcohol and a little ammonia, absorbs this fluid by blotting-paper, and then examines in dilute glycerine, in which material the preparation may also be preserved if the cover-glass is ringed with a border of varnish. Preparations can also be preserved in glycerine-gelatine (pp. 213–14).

For staining moulds basic aniline dyes are used, e.g. for sections Loeffler's methylene blue.

Detection of arsenic by moulds (Abel-Buttenberg, *Zsch. f. Hyg.*, vol. 32). The substance to be tested is mixed in a flask with a large proportion of brown bread crumbs, after neutralizing if necessary. Sterilize in steam and inoculate with Penicilium brevicaule.

Close the flask tightly with two rubber caps over the plug. When after 24–48 hours' incubation at 37° C. the mould has grown freely : the smell of garlic in the flask indicates the presence of arsenic.

The skin parasites (favus, tricophyton, &c.) can be cultivated on gelatine or on agar, and may be stained in the same way as bacteria. When isolating them, it is advisable to pound up the crusts, hair, &c., in a sterilized mortar with infusorial earth previously sterilized by heating it till it glows and then allowing it to cool. From the material crushed up in this way make plates and subculture from colonies which the microscope shows to have clearly developed from a single spore. Perhaps before it is sown, the material may be placed in 50 % (or stronger, up to absolute alcohol) for 1–24 hours. Under this treatment the bacteria are entirely, or for the most part destroyed, whereas the fungus for the most part survives. Very simple and successful is the following method of cultivation *in situ* devised by Plaut. One puts a diseased hair or piece of epidermis on a sterilized slide, firmly presses on it with another sterilized slide, and then draws the slides asunder and covers the material with a sterile cover-glass and fastens the edge with wax. Never add water. Place the preparation in a damp chamber (so damp that the label stuck to the slide is easily detached when 24 hours is up). The fungi grow well under these circumstances. After a few days, subculture from the edge of the proliferating fungus on to gelatine or agar plates.

24. Amoebae.

Cultivation of some kinds of amoeba (e.g. from water, earth, or the intestine) is possible. The following media may be used:—

1. *Hay or Straw Media.* 20–40 grams of hay or straw are boiled with 1 litre of aq. com. for half an hour. Make the filtrate slightly alkaline with sodium carbonate, boil, filter. A solid medium can be obtained by adding $1\frac{1}{2}$ % of agar before neutralization.

2. *Fucus Crispus Medium.* To broth or to tap-water is added 5–15% of fucus crispus and the medium boiled until it is dissolved (takes a long time). The medium is then rendered alkaline (1 c.c. of decinormal potash to 10 c.c. of the medium).

3. The ordinary culture media may be used, and also the following combination, agar 0·5, tap-water 90, ordinary alkaline nutrient broth 10.

It is not possible to obtain pure cultures, since bacteria upon which the amoebae nourish themselves must also be always present. If such a mixed culture, in which no bacterial spores are present, or moulds, or yeasts, and in which the amoebae have formed cysts, is treated for seventy-two hours with 20% solution of water-free soda (see p. 16), the bacteria die out. The amoebal cysts do not then grow on ordinary culture media, but can do so if they are transferred to pure cultures of bacteria capable of serving as food for them. (In this way a culture of one kind of amoeba with one kind of bacterium may be obtained.)

Amoebae are best examined in hanging-drop preparations with 0·8 % NaCl solution. For staining fix

in sublimate alcohol (saturated aqueous sublimate solution two parts, absolute alcohol one part). Preparations, still moist, should be dipped in for a few seconds and then washed for half an hour in 60% alcohol to which sufficient iodine has been added to produce a brownish colour; they are preserved in 70% alcohol. Before staining, wash in water. Stain with picrocarmine ½-1 hour in the cold, wash out carefully in 70% alcohol containing 0·1% HCl. Counter-stain with weak aqueous solution of light green, transfer to absolute alcohol, cedar oil, &c. Pieces of tissue (e.g. intestine in tropical dysentery) are fixed in sublimate alcohol ½ hour, washed in iodine alcohol, as above, 24 hours, transferred to absolute alcohol, xylol, and embedded in paraffin. Sections are stained in the same way as fresh preparations (see above). Giemsa's stain may also be used (see p. 173).

25. Malaria Parasites.

For preparation of blood films see p. 181. If the parasites are scanty the following procedure may be followed. Spread out a large drop of blood on a cover-glass, allow it to dry in the air, and then place it for several minutes in a mixture of equal parts of 2% formalin and ½-1% acetic acid. By this means the haemoglobin is dissolved out. Stain by one of the following methods:—

a. Simple staining according to Manson.

Dissolve pure methylene blue (Höchst) 2, borax 5, in boiling water 100. Before use dilute this stain with water until it just appears transparent in a test-

tube. In the case of freshly-prepared dry films, staining in the cold for 10–15 seconds suffices; but older preparations are better stained with methylene blue 1, crystallized soda 0·2, aq. 100, for a few up to 20 seconds without applying heat. Wash in water. Parasites blue, red corpuscles greenish.

b. Double staining by Loeffler's method (see p. 111).

c. Chromatin stain according to Giemsa (a modification of the Romanowsky-Nocht method). (*Centr. f. Bakt.*, Abt. I, Or. 37, p. 308.)

1. Fix the preparations in ethyl alcohol for 15-20 minutes, or (only for 2–3 minutes) in methyl alcohol. Remove the fluid by blotting-paper.

2. Stain with Giemsa's solution for Romanowsky stain, which can be obtained from Grübler, Leipzig. Fill this stain into a drop bottle (which before use must be rinsed out with absolute alcohol). To some water which has been warmed in a test-tube to 30–40° C. add, while it is being shaken, 1 drop of the stain for each cubic centimetre of the water. (The freshly diluted stain is run on to the cover-glass preparations and allowed to act from 10–15 minutes; the solution should be renewed once.)

Note—all vessels must be scrupulously clean and free from all trace of acid.

3. Wash in running water.

4. Dry, and mount in immersion oil.

The chromatin of the parasites is shining red, protoplasm blue, leucocyte nuclei red to violet, their protoplasm blue; red corpuscles rose to brownish red.

d. Giemsa's rapid staining method. (*M. m. W.* 1910, p. 2476.)

1. Very thin air-dried films on slides are laid, film side uppermost, in a dry Petri dish; 10–15 drops of the solution described (under *c.* 2 above) are mixed with an equal volume of pure methyl alcohol and poured on the slides. After 30 seconds

2. Pour so much distilled water into the dish as will suffice to cover the slide completely (10–15 c.c.) and mix the stain and water thoroughly by rocking. Leave to stand 3 minutes or longer.

3. Pour off the liquid, wash in running water, dry, and mount in cedar oil.

Staining of sections by Giemsa's method. (*D. m. W.* 1910, No. 12.)

1. Pieces of tissue of not more than 5 mm. thickness are fixed in sublimate alcohol (see above under Amoebae) for at least 48 hours, the fluid being renewed after 24 hours. All manipulations to be carried out with horn forceps, &c. Prolonged stay in the solution does not harm the subsequent staining.

2. Transfer to increasing strengths of alcohol, xylol, and embed in paraffin. Sections are cut and fixed to slides in the usual way, and taken down through xylol and the alcohols to water.

3. Place for 10 minutes in potassium iodide (2% in water) 100 c.c., Lugol's iodine-potassium iodide solution 3 c.c.

4. Wash in distilled water and place for 10 minutes in $\frac{1}{2}$% thiosulphate solution, wash 5 minutes in tap-water and rapidly in distilled water.

5. Stain in Giemsa's solution (see above—for prolonged staining use 1 drop to 2 c.c. instead of 1 c.c.) for 2–12 hours. Renew the stain after $\frac{1}{2}$ hour.

6. Wash in distilled water and take through acetone xylol mixtures (acetone 95, xylol 5; acetone 70, xylol 30; acetone 30, xylol 70) to xylol; mount in cedar oil.

The various elements are stained as above (*c.* 4).

26. Trypanosomes.

Certain varieties can be cultivated in the condensation water of a mixture of equal parts of nutrient agar and defibrinated rabbit's blood. Sow richly, keep at 37° C.

They may be examined whilst alive in hanging drop with 0·8 % salt solution or spread out between a slide and coverslip (ringed round with vaseline or wax).

Blood films containing trypanosomes can be stained by Giemsa's method (see p. 172, *c.*, stain 2–5 minutes) or according to Loeffler as follows:—Prepare thin films and fix with equal parts of alcohol and ether. Stain on the coverslip with three drops aqueous 0·5% sodium arsenate solution and one drop 0·5% aqueous malachite green (Höchst), warm until steam rises 1 minute. Wash in running water. Into a test-tube put 5 c.c. of 0·5 % glycerine puriss. in water, add to it 5–10 drops of Giemsa's stain, heat to boiling and whilst hot pour over the coverslip. Pour off after 1–5 minutes (the Giemsa-glycerine mixture can be used again) and wash in a strong stream of water. Protoplasm of the trypanosomes blue; nuclei, undulating membrane and flagella red, red corpuscles rose coloured.

Moist preparations and sections can be stained by Giemsa's method.

27. Spirochaetes (Treponema) of Syphilis.

The spirochaetes of syphilis are distinguished from other kinds by the fact that their staining capacity is, comparatively speaking, less pronounced, and also by their extraordinary fineness and by their shape. They possess especially numerous (10–26) regular deep corkscrew-like convolutions and both ends are very pointed. In the living state they can readily be observed by means of dark-ground illumination (see page 6); in the unstained state they can also be seen by mixing a drop of fluid containing them on a coverslip with Indian ink and making thin films (see p. 38).

Multiplication occurs on solidified horse-serum anaerobically but pure cultures have not yet been obtained (Mühlens, *Klin. Jahrb.* 23, p. 339).

Detection for diagnostic purposes is best accomplished in films from untreated chancres, papules, &c. The lesion is well cleaned with cotton-wool and petroleum and ether; by firm pressure, or if necessary by scratching with a coverslip, some serum is obtained as free from blood as possible and spread on a slide. For staining smear preparations, instead of the usual methods in which the stain must act for hours (the organism is Gram-negative) the following are used:—

a. Giemsa's method (*D. m. W.* 1905, No. 26; 1907, No. 17):

1. Fix in alcohol or carefully in the flame. In old preparations fixation may be omitted.

2. Stain (as directed on page 172, *c.*) 30–60 minutes without warming (the water used for diluting the stain

should be made alkaline by the previous addition of 1–10 drops of 1% K_2CO_3 solution).

3. Wash quickly in water free from acid.

The spirochaetes stain an intense deep red, background faintly reddish or colourless, cells blue, nuclei red, erythrocytes rose.

b. Giemsa's rapid staining method (see p. 172).

c. Loeffler's method: as for trypanosomes (see p. 174).

For staining in sections one employs:—

Method of Levaditi (Hoffman, *D. m. W.* 1906, No. 22).

1. Slices of tissue not more than 2 mm. thick are fixed for 24 hours in 10% formalin in water.

2. Transfer to 96% alcohol 15 hours.

3. Transfer to distilled water until slices sink, change several times.

4. Suspend the pieces on silk threads in a freshly-prepared mixture of 90 c.c. 1·5 % $AgNO_3$ and 10 c.c. purest pyridine in a dark bottle with a glass stopper. The tissue is left in this solution up to six days.

5. Transfer, after washing in 10% pyridine solution, to a freshly-prepared mixture of 90 c.c. 4% aqueous pyrogallol solution and 10 c.c. acetone; to 85 c.c. of this mixture are added 15 c.c. pyridine. The solution is kept in a dark glass-stoppered vessel and the tissue is left in 15–48 hours. (It is best to wash the tissue once or twice with the solution in a watch-glass in the dark before putting it into the glass jar.)

6. Wash in water, pass through the alcohols to xylol, embed in paraffin, cut, fix to slide, and examine without further staining. Spirochaetes are stained black by the silver.

28. Spirochaetes of Relapsing Fever.

The spirochaetes may be examined alive or stained by the methods used for syphilis. The coils are less deep. The organism is plentiful in the blood during an attack. Cultivation has not been successfully carried out. On the other hand, Spirochaeta Dentium grows anaerobically at 37° C. in a mixture of horse-serum one part to two parts of nutrient agar with mechanical exclusion of air (see p. 41). Granular colonies are formed after about ten days.

29. Rabies Granules.

Negri's bodies are sought after the method of Lentz (*Centr. f. Bakt.*, Or. 44, p. 374).

1. Transverse sections of the Cornu Ammonis some 2–3 mm. thick are fixed, hardened and embedded by Henke-Zeller rapid method (see p. 64). Sections are fixed to the slide, the paraffin is removed by xylol and the latter by absolute alcohol for one minute.

2. Stain in a solution of eosin extra B. Höchst 0·5 % in 60 % alcohol.

3. Wash in water.

4. Stain in Loeffler's methylene blue 1%.

5. Wash in water, dry with blotting-paper.

6. Differentiate in absolute alcohol 30 c.c., to which have been added 5 drops of 1% alcoholic NaOK, until the colour is pale rose.

7. Differentiate in absolute alcohol 30 c.c. + 1 drop 0·50 % acetic acid until the ganglion cell layers appear as faint blue lines only.

8. Wash rapidly in absolute alcohol, xylol, mount

in balsam. Negri's bodies are carmine red, their inner granules blue, ganglion cells light blue except the nuclei, the nucleoli blue black, red blood cells vermilion.

One can also cut out the ganglion cells with a scalpel from a fresh section of the Cornu Ammonis, crush between two slides, fix the films whilst still wet for a few minutes in methyl alcohol, wash in absolute alcohol and stain as above.

VII

Methods of obtaining Material from the Body for Bacteriological Examination

(Revised and amplified by Dr. T. J. Horder, F.R.C.P.)

VARIOUS secretions, pathological exudates, and other fluids which are to be examined bacteriologically are removed and preserved in such a manner that (1) they do not become contaminated by extraneous micro-organisms, and (2) they do not come in contact with disinfectants.

(I) **Blood.** *A.* Small quantities of blood, such as are required for Widal's test or other agglutination reactions (see p. 133), the technique of phagocytic or opsonic indices (see p. 198), the investigation of bactericidal action (see p. 194), or the preparation of culture media, may be best withdrawn from the skin at the dorsal surface of the terminal phalanx of the finger. The finger is scrubbed with

soap and water for some minutes and is then rubbed with sterile wool soaked in equal parts of absolute alcohol and ether. The cleansing process ensures disinfection of the skin and also induces a good flow of blood to the part. The blood flows more freely from the puncture if an elastic band is wound round the proximal part of the finger immediately before the puncture is made. This object is also ensured by swinging the arm well before the elastic band is used. It is also further ensured by flexing the terminal joint of the finger. When a small lancet or bayonet-pointed needle has been sterilized in the Bunsen flame, a little alcohol is poured over the finger and allowed to evaporate. The puncture is then made. If these directions be carried out a considerable amount of blood may easily be obtained. The blood is collected in capillary tubes, the sealed ends of which are broken off by sterile forceps and passed through the flame, to be sealed after the blood has been drawn. The size of the tube varies with the amount of blood which it is required to collect. The puncture wound may be closed by application of a little collodion.

If the *serum* is required, this is allowed to separate by standing the blood in the ice-chest; or its separation is promoted by centrifugalizing. The tube is then broken by sterile forceps, and the end of a fine glass pipette is introduced into the serum, which rises into the tube to any measured distance. From this tube the serum may be transferred to any required vessel, and, if necessary, diluted to any desired degree.

B. Larger quantities of blood, such as are required for purposes of *blood-culture*, are best obtained by

puncturing a good-sized vein at the bend of the elbow with a sharp, clean hollow needle attached to a glass syringe capable of holding 5 or 10 c.c. Needle and syringe are boiled in distilled water immediately before use. The skin over the vein is cleansed as in *A*, the arm is allowed to hang down fully extended, and, if the vein is not already rendered prominent by this means, a bandage is tied round the arm near the shoulder. It is advisable to draw up into the neck of the syringe a little of a 0·5 % sterilized solution of sodium citrate to prevent clotting in the needle. The needle is then inserted into the vein, care being taken to hold the syringe as far as possible parallel to the surface of the arm: by this means puncture of the opposite wall of the vein is avoided. If the needle is in the vein, the blood will enter, and will often fill the syringe without suction being employed ; no more suction than is necessary to fill the syringe in a reasonable time must be used, as this tends to introduce air at the junction of the needle and syringe. This method of obtaining blood is by far the best when it is required for purposes of determining the presence of micro-organisms in it, because the blood at no time comes into contact with the skin or with the air. The syringe being full, the needle is quickly withdrawn, a finger being placed upon the puncture wound whilst an assistant removes the bandage and raises the arm. The puncture then ceases to bleed, and may be sealed with a little collodion. For purposes of blood-culture the blood is now squirted into tubes of various media, and these are incubated at 37° C. The syringe being graduated, definite amounts of the blood can be

added to broth tubes, or sown on solid media, and thus a quantitative estimation of the organisms present in the blood may be obtained. For purposes of preparing blood-media for the growth of haemophilous organisms, proceed as on p. 162.

C. Blood from the cadaver is withdrawn from the heart by searing the surface of the right auricle with a hot iron, and inserting the unsealed end of a stout glass pipette, or the needle of a syringe—either having been previously sterilized.

D. To make a blood preparation for direct microscopical examination, the margin of a cover-glass, which has been sterilized in the flame and allowed to cool, is placed against a drop of blood as it oozes from the puncture wound. With the edge of this cover-glass the surface of several clean slides or cover-slips are then streaked, each with a single stroke, The layer of blood thus deposited should be even and thin. The films are allowed to dry in the air, being covered by watch-glasses in the meantime. They are placed for fixation in absolute alcohol, or in equal parts of alcohol and ether, whence they may be removed for staining at any time from 15 minutes to 24 hours. (Fixation in the flame is apt to damage the red blood corpuscles.)

(II) **Cerebrospinal fluid.** *A.* This is obtained during life by means of *lumbar puncture.*

In performing the puncture the patient lies upon his side, with knees drawn well up, head bent down, and spine arched with its convexity backwards. Some opisthotonos incidental to the disease may render this position impossible, and the puncture accordingly more difficult. The skin over the lower lumbar spine

is thoroughly scrubbed with soap and water, and is then treated with ether. Meanwhile, a syringe and a stout needle are being boiled close by.[1] The needle used for an adult should be $3\frac{1}{2}$ inches long; for an infant it should be 2 inches long. The spinous process lying nearest to the line joining the highest points of the iliac crests is that of the fourth lumbar vertebra. The puncture is made midway between this spine and the one next above or below it. In an adult a point is chosen $\frac{1}{2}$ inch, in an infant somewhat less than $\frac{1}{2}$ inch, from the middle line. The direction of the needle in an adult is important: it should be forwards, inwards, and upwards. In infants the direction may be almost straight forwards. The needle is pushed firmly onwards until the gap between the laminae is entered, and fluid flows from the needle. If the course of the needle seems obstructed it must be withdrawn a short distance, any faulty direction corrected, and a fresh attempt be made. Suction by the piston is not required; if the needle is in the spinal canal and its orifice is patent any fluid present will flow, whether there be meningitis or not. A dry puncture may mean that the needle has not entered the canal, or that it has struck a nerve root, or that the pus present is too inspissated to flow. The first is the most usual explanation.

B. From the cadaver cerebrospinal fluid may be obtained by lumbar puncture, or it may be collected from the ventricles of the brain or from the distended pia-arachnoid membrane by sterile glass pipettes, which are sealed immediately afterwards.

[1] The trochar and cannula sold with Barker's Spinal Anaesthesia Syringe is especially to be recommended for lumbar puncture.

(III) **Pus** and various **pathological exudates** are best withdrawn by a sterile syringe, after thorough disinfection of the skin. The syringe is taken to the laboratory, or the material is transferred from it to sterile test-tubes, or is sown directly into tubes containing sterile water, from which cultures may be made later upon appropriate media.

(IV) **Secretion or membrane from the throat** (Cases of sore throat, suspected diphtheria, &c.). With the patient's mouth well illuminated, and the tongue depressed, touch the diseased part with a sterilized platinum loop or with a plug of sterile wool fixed to a wooden rod or wire (i.e. a 'swab'). It is an advantage to keep these throat swabs in a sterilized tube, the handle of the instrument projecting so that it can be readily grasped. Such swabs are obtainable from most bacteriological laboratories. In removing the material, care must be exercised that the affected part of the throat is really touched—if any doubt arises, the operation must be repeated. After touching the part, the swab or loop is replaced in its tube, unless the examination can be made forthwith. Care should be taken to prevent the material from drying up, as some bacteria (e.g. meningococcus) rapidly perish under the influence of desiccation. For exact determination of the kinds of bacteria present, and their relative abundance, it is essential to make quantitative dilutions of a known amount of the material (e.g. a standard loopful of it). *Nasal secretion* is removed in a similar manner; the operation is facilitated by the use of a speculum and a head mirror.

(V) **Sputum** should be obtained as free from food

and from saliva as possible. It should be expectorated directly into a clean vessel, which, of course, contains no disinfectant. In making the necessary examinations care must be exercised in selecting portions of the sputum most likely to give positive results. Thus, if the question of tubercle bacilli is under investigation, any caseous material present should be examined; if the pneumococcus or the influenza bacillus is being sought for, any 'rusty' or blood-stained mucus should be dealt with; if actinomycosis is suspected, any 'granules' present must be isolated. A hand lens is often of service for the initial examination.

(VI) **Urine.** A sterilized catheter is passed, the first portion of urine is discarded, and the later portion is received into a sterilized flask, which is then plugged with wool.

(VII) **Faeces.** These are received into a clean vessel without disinfectant. The use of a hand lens is again of service. When mucous flakes are to be examined the stool may well be transferred to sterile water.

VIII

Methods of examining the Blood in relation to Immunity

For particulars as to the quantitative estimation of antitoxin in serum see p. 113; agglutinin, pp. 123, 133, 146; bacteriolysin (Pfeiffer's test in reference to typhoid and cholera), pp. 118, 147.

1. *The original method of Bordet and Gengou of demonstrating the presence of 'Substance Sensibilisatrice' (Amboceptor) in samples of immune serum by the Absorption of Complement* (*A. I. P.*, xv, No. 5, May, 1901).

This method is an application of the following two facts established by Bordet, viz.:—(1) Red blood corpuscles or bacteria when sensitized (i.e. when united in vitro with 'substance sensibilisatrice' of immune serum) acquire the power of rapidly absorbing complement; (2) In one and the same serum, complement can provoke either haemolysis, or, in the case of certain bacteria, bacteriolysis.

The procedure consists in bringing together immune serum, bacterial emulsion, and complement. A control experiment is made in the same way with normal serum. In presence of the immune serum, the microbe becomes sensitized and absorbs any complement that may be present, whereas in presence of the normal serum no such absorption occurs. Accordingly, after the mixtures have been allowed to stand for a sufficient time, complement is tested for by adding sensitized red blood corpuscles to each. In case of the normal serum these sensitized red blood corpuscles rapidly become haemolysed; but in case of the immune serum, provided it contained 'substance sensibilisatrice', no haemolysis takes place.

Bordet and Gengou carried out this test in the instance of the plague bacillus in the following way:—

Materials employed. (1) Serum from a horse immunized against plague (heated to 56° C. for $\frac{1}{2}$ hour to destroy complement). (2) Serum from a normal horse heated in the same way. (3) Twenty-four hours'

agar culture of plague emulsified in a small quantity of normal saline. (4) Serum freshly drawn from a normal guinea-pig and freed from corpuscles by centrifugalization: this is the complement.

Admixture. Tube (*a*) contains $\frac{2}{10}$ c.c. complement, $\frac{4}{10}$ c.c. plague emulsion, $\frac{12}{10}$ c.c. anti-plague serum.

Tube (*b*) $\frac{2}{10}$ c.c. complement, $\frac{4}{10}$ c.c. plague emulsion, $\frac{12}{10}$ c.c. normal horse serum.

Tubes (*c*) and (*d*) are the same as (*a*) and (*b*), but without the plague emulsion.

Tubes (*e*) and (*f*) are the same as (*a*) and (*b*), but without the complement.

Test for Complement. After these tubes have stood for five hours at the laboratory temperature (15–20° C.) each of them receives two drops of blood very strongly sensitized (i.e. each receives $\frac{2}{10}$ c.c. of the following mixture:—2 c.c. of serum (previously heated to 56° C.) derived from a horse that has received 3–4 injections of 4–5 c.c. defibrinated rabbit's blood; 20 drops of defibrinated rabbit's blood).

Result. Haemolysis was found to take place rapidly in tubes (*b*) (*c*) (*d*)—in about 5–10 minutes these tubes had no more red blood corpuscles intact. In tube (*a*) haemolysis did not take place.

Conclusions. (1) B. pestis mixed with serum of a normal horse does not absorb complement. (2) The same bacillus in presence of anti-plague serum from a prepared horse fixes the complement and deprives the fluid of it. (3) Anti-plague serum to which the bacillus has not been added leaves the complement free.

2. *Quantitative estimation of complement.*

Complement disappears from serum when the latter is exposed to a temperature of 55° C. for thirty minutes. Its abundance, accordingly, can be measured by exposing a sample of immune serum to this temperature, thus inactivating it, and then determining the smallest quantity of the serum of test required to reactivate this immune serum. Typhoid and cholera immune sera are especially suitable for this purpose owing to their marked bactericidal properties.

3. *Serum Diagnosis of Syphilis.*

(Wassermann's Reaction, worked out by Wassermann, Neisser and Bruck.)

This reaction is the application of the Bordet-Gengou Fixation of Complement test (p. 185) to Syphilis. The following description is taken from the fifteenth edition of Professor Abel's book.

Principle: In contact with extracts of syphilitic tissues the serum of a syphilitic patient, inactivated by warming, fixes the complement of normal guinea-pig serum, and thus prevents its bringing about the solution of the haemoglobin when added to a mixture of red corpuscles and their inactivated appropriate haemolytic serum.

Certain substances, to which the general term 'Antigen' is applied, e.g. bacteria, toxins, blood-corpuscles, proteins, &c., when injected into the bodies of appropriate animals provoke the formation in their serum of substances called 'Antibodies'. These antibodies act only on the antigen which called for their production, not on other antigens, and show

their activities in various ways, e.g. antitoxins, haemolysins, agglutinins, &c. Antibodies of this type arise in the human body or in animals on their recovery from a natural infection, e.g. the agglutinin in the serum of a typhoid patient (see Widal's Reaction).

On warming to 56° C. many of these antibodies lose their activity but recover it on the addition of fresh serum of a normal animal. For example, in the serum of rabbits which have been treated by the injection of sheep's corpuscles (antigen) there arises an antibody in the form of haemolysin, so that the serum of such rabbits, even in high dilution, dissolves sheep's corpuscles and sets free the haemoglobin. If the rabbit serum is heated to 56°C. for half an hour it fails to dissolve the sheep's corpuscles and is said to have become 'inactive'. If now to a mixture of this heated serum and sheep's corpuscles one adds a small quantity of fresh serum of a normal animal, preferably a guinea-pig, solution of the corpuscles (haemolysis) occurs.

The term 'amboceptor' is applied to the inactivated antibody in the serum of the immunized animal (in the case given the inactivated serum of rabbit injected with sheep's corpuscles); the normal guinea-pig serum whose addition activates the amboceptor is called 'complement'. The mixture of sheep's corpuscles, amboceptor and complement in the example given, is termed a 'haemolytic system'. The addition of amboceptor to the sheep's corpuscles is called 'sensitizing' the corpuscles.

When an antigen is mixed with an appropriate inactivated antibody (amboceptor) and complement

added, the three substances form a firm combination; the complement does not remain free (disposable) but is bound (fixed). So, for example, if typhoid bacilli are mixed with the inactivated serum of an animal which has been treated by injection of typhoid bacilli, and some fresh guinea-pig's serum added as complement, the complement is fixed after the mixture has stood for a short time. If now one adds sensitized sheep's corpuscles (i. e. sheep's corpuscles and their appropriate amboceptor, the inactivated serum of a rabbit which has previously been injected with sheep's corpuscles) no haemolysis occurs, for the necessary complement has been previously used up by being fixed to the typhoid bacilli and the inactivated typhoid serum. On the other hand, if one mixes typhoid bacilli, inactivated cholera serum (heated serum of an animal previously injected with cholera vibrios) and complement together, and, after leaving the mixture to stand for a short time, adds sensitized sheep's corpuscles, haemolysis occurs because typhoid bacilli and cholera-serum do not correspond to one another, do not undergo combination, and so leave free the complement for the blood corpuscles.

It is clear that this method of experimentation, when closely confined to definite quantitative proportions in the mixtures, can be used to test whether a known antigen corresponds to an unknown serum as amboceptor or not, and contrariwise. If haemolysis does not take place the antigen and serum correspond, if there is haemolysis they do not. If one brings together an extract of a syphilitic organ (antigen) and human serum inactivated by warming, adds comple-

ment, and after a time sensitized sheep's corpuscles, haemolysis does not occur when the subject is infected with syphilis, i.e. has syphilis antibodies in the serum.

Technique of the reaction. The following are required:—

1. As antigen, an aqueous extract of syphilitic liver (to be obtained with particulars of the amount to be used from the Universitätsklinik für Hautkrankheiten, Breslau, from Merck, Darmstadt, or Messrs. Zimmermann, London). For practical purposes, instead of this an alcoholic extract of normal organs (guinea-pig's heart and normal human liver) may be used, but in doubtful cases the reaction must always be tested with aqueous extracts of syphilitic organs. It is advisable at first to test every serum with several extracts, when it must react with all if the diagnosis of syphilis is to be made.

2. The inactivated body fluid to be examined (blood serum, lumbar puncture or ascites fluid), 2–3 c.c. are desirable. The inactivation should be performed by heating to 56° C. in an oven or water-bath for ½ hour as soon as possible after the material has been collected; in particular, blood serum should be separated off and inactivated immediately after coagulation. When preserved sterile the inactivated fluids keep in good condition for the reaction for a considerable time.

3. As complement the fresh unheated serum of a normal guinea-pig (freed from corpuscles by the centrifuge) is used. Usually the serum remains active for a day only, but when frozen (in a freezing mixture) it keeps longer.

4. A suspension of washed sheep's corpuscles. Fresh

defibrinated sheep's blood is centrifuged, after the point to which the tubes are filled has been marked with a grease pencil. The serum is pipetted off and replaced by 0·85 % salt solution. The suspension is again centrifuged and the washing is repeated four times. Finally salt solution is added up to the mark. For use 5 volumes of this strong suspension are mixed with 95 of 0·85 % salt solution.

5. Haemolytic amboceptor in the form of serum of a rabbit immunized to sheep's corpuscles. 30 c.c. of a suspension of washed sheep's corpuscles, prepared as above, are injected into the peritoneal cavity of a rabbit. Blood removed from a vein of the ear 8–10 days later already yields an active serum, but it is better at this stage to make another injection of the same dose of corpuscles, and to remove blood not earlier than ten days later. The serum must be perfectly clear (centrifuge if necessary); it is warmed $\frac{1}{2}$ hour to 56° C. When kept cool and in the dark it keeps for a long time, but its strength must be tested before every complement fixation experiment. (It can be obtained from Merck, Darmstadt, or Zimmermann, London.)

6. Serum of a syphilitic patient which gives a definite Wassermann reaction.

7. Serum of a healthy individual which does not give a Wassermann reaction.

In a preliminary experiment the titre of the haemolytic system is determined. Diminishing quantities of serum (see 5 above) are mixed with 1 c.c. corpuscle suspension (4) and 1 c.c. of a 1 in 10 dilution of serum (3) in 0·85% salt solution, and the mixtures are placed

in the incubator at 37° C. In carrying out the test one uses two to three times the minimum quantity of serum (5) which just suffices to dissolve the corpuscles within two hours (i. e. so that the tube contains a clear red fluid without sediment).

Further, by means of a preliminary experiment with a definitely syphilitic serum one determines how much antigen (1) it is desirable to use.

In the experiment six tubes are used.

In tube 1 is placed 1 c.c. of a 1 : 5 dilution in 0·85 % salt solution of the fluid to be examined (2). To it is added the quantity of antigen indicated by the preliminary experiment also diluted to 1 c.c. with salt solution.

In tube 2 is placed 1 c.c. of a 1 : 5 dilution in saline of a known syphilitic serum together with antigen as in tube 1. (This tube serves to control the activity of the antigen.)

In tube 3 is placed 1 c.c. of a similar dilution of a serum which is definitely not syphilitic, together with antigen as in tube 1. (Control for the utility of the antigen.)

In tube 4 is placed double the amount of antigen in tube 1, made up to 2 c.c. with salt solution. (Control to show that the antigen does not of itself inhibit haemolysis.)

In tube 5 is placed double the amount of the fluid to be tested that has been placed in tube 1, made up to 2 c.c. with saline. (Control that the fluid to be tested does not of itself prevent haemolysis.)

In tube 6 are placed 2 c.c. of the saline solution used (control of the proper composition of the hae-

molytic system). To each tube is then added 1 c.c. of a 1 in 10 dilution of the complement (3) in saline solution, so that now each tube contains 3 c.c. fluid. The tubes are placed in the incubator at 37° C. for one hour.

After this time one adds to each tube two or three times the minimal quantity of inactivated haemolytic amboceptor (5) determined in the preliminary experiment, and 1 c.c. of the 5% suspension of sheep's corpuscles (4). The tubes are well shaken and replaced in the incubator. The tubes are examined from time to time; the experiment is complete when in the sixth tube, which contains only the haemolytic system and saline solution, complete solution of the corpuscles has taken place. If the serum to be examined is derived from a syphilitic patient the corpuscles must remain undissolved in tubes 1 and 2 (the fluid must be uncoloured and there must be a red deposit); in all the other tubes there must be complete haemolysis (fluid clear red, no deposit).

Should the patient from whom the serum was obtained have shortly before suffered, or be at the time suffering from scarlet fever, malaria, or leprosy, the value of the result is diminished. In general, a positive reaction indicates lues, but a negative reaction never excludes it. Where the reaction is weak, i.e. where haemolysis is incomplete or sets in late, it is advisable to repeat the examination after a few weeks. Diminished haemolysis after this time favours the diagnosis of syphilis. The reaction is positive in manifest syphilis in about 95 %, in the quiescent stages in about 50 %, in paralysis in approxi-

mately 100 % of cases. The effect of specific treatment of syphilis, especially in recent infections, manifests itself by the disappearance of the reaction (i.e. by the occurrence of haemolysis in the test).

4. *Methods of estimating the bactericidal power of blood.*

(*a*) *Test-tube method.* The experiments are made in small test-tubes in which defibrinated blood, serum, washed corpuscles, &c., in definite proportions are brought in contact with living bacterial suspension. Cultures are made from measured quantities of the mixture (e.g. by a loop) directly, and after various intervals at 37° C., and the number of living bacteria present determined. It is desirable that thorough admixture should be obtained, and that apparent decrease in the number of bacteria due to agglutination should not be confounded with decrease due to bactericidal action.

(*b*) *Wright's method of determining the bactericidal value of small samples of blood* (*P. R. S.*, lxxi, No. 468, October, 1902).

The method consists in bringing a series of measured volumes of undiluted serum into contact with a series of graduated dilutions of a culture of the microbe of test, the object being to determine the lowest dilution of the culture upon which a complete bactericidal effect is exerted by the serum.

Dilution of the culture is effected by a special pipette graduated from 2·5 cm. to 250 cm., and directions for its construction are given in the paper referred to. The pipette is worked by means of a rubber teat fixed on its upper extremity. The highest

dilutions of the culture are cultivated on agar and the number of bacteria present determined.

For mixing equal volumes of serum and graduated dilutions of the culture a special form of capillary testing pipette is used, and a feature of this pipette is that its bulb subsequently serves as a culture chamber. The upper portion of the pipette presents a spiral twist—an ingenious device for keeping out air bacteria—and at its upper extremity a rubber teat is fixed. When operating the pipette, the first stage is to fill its bulb $\frac{3}{4}$ full of sterile broth—the *lower* quarter being free. Equal volumes of serum and of culture dilution are then measured off, mixed, drawn into the capillary end of the pipette and allowed to remain at the requisite temperature for the allotted time. The capillary end of the pipette is then drawn out into the finest possible filiform tube, a condition of negative pressure is established in the interior of the pipette by fitting on its upper end a collapsed rubber teat, and this negative pressure carefully regulated by keeping the finger and thumb in position on the teat. The filiform tube is then gently snapped across, and the column of fluid gradually mounts into the broth contained in the bulb of the pipette. The pipette is now incubated, and the question of growth determined.

5. *Leishman's method of determining the phagocytic power of the blood* (*B. M. J.*, January 11, 1902).

A bacterial suspension, of appropriate density, is prepared with normal saline solution and the following apparatus got ready. 1st, an ordinary microscopic

slide and some cover-glasses; 2nd, a 'moist chamber', prepared by laying a piece of damp blotting-paper at the bottom of a Petri dish; 3rd, a capillary pipette, furnished with a rubber teat and marked on the stem with a grease pencil. The finger of the patient is now pricked and a drop of blood drawn up to the mark on the pipette, this volume is allowed to run up the tube for about a quarter of an inch and a similar volume of the bacterial suspension is then drawn up after it. Both volumes are then expelled from the tube into a clean watch-glass and well mixed by being drawn in and out of the tube several times. A small drop of the mixture is now placed at one end of the microscope slide and quickly covered with a cover-glass. This procedure is next repeated with the blood of the normal individual who is to serve as a control, usually that of the observer himself, and the drop of this mixture is placed at the end of the same slide and similarly covered with a cover-glass. The slide is now placed in the incubator, at a temperature of 37° C. for fifteen minutes, being first put into the 'moist chamber', resting upon two wooden matches. At the end of this time it is removed and the films prepared as follows. The condensation water is wiped from the under side of the slide—none will be found on the upper surface—and the cover-glasses are smoothly slid off the slide with the help of the points of a pair of forceps. Should they be found to have adhered to the slide at their edges a drop of saline may be run round the edges of the glass which will soon loosen the connexion and will not be found to interfere with the films. The films left on the slide,

when dry, are then stained, the cover slips being held in reserve, either by Leishman's stain or by any other method which will demonstrate the ingested bacteria with sufficient clearness. The bacteria taken up by each polynuclear leucocyte of the patient's blood are now enumerated, 50 cells at least being counted, the average number ingested is ascertained and this figure is contrasted with the average number taken up by the leucocytes of the normal control blood. The result is expressed as the ratio found to exist between the two.

Modification effected by Wright and Douglas (*P.R.S.*, lxxii, No. 483, October, 1903).

W. and D. modified the method of Leishman (*a*) by conducting the phagocytosis in capillary tubes, making afterwards film preparations in the ordinary way; (*b*) by decalcifying the blood with citrate of soda, thus avoiding the complications introduced by blood coagulation, and making it possible to separate the white corpuscles from the blood fluids by centrifugalization, decantation, and washing. The procedure practised by W. and D. when comparing the phagocytic power of different samples of blood is described by them as follows:—

Procedure No. 1, *employed where nothing more than a comparison between bloods from different sources, or bloods subjected to different conditions, is required.*

Having provided ourselves with a simple capillary pipette, furnished with a rubber teat and a pencil mark on the stem, we aspirate into the stem of the pipette—dividing off by bubbles of air in accordance

with the procedure introduced by one of us—one volume of blood from the finger, one volume of a 1-per-cent.-solution of citrate of soda in physiological salt solution, and one volume of a bacterial suspension made by shaking up a 24-hour agar culture in physiological salt solution and centrifugalizing so as to remove any bacterial clumps. We mix together the three equal volumes of blood, bacterial suspension and citrate of soda solution, by blowing these out upon a clean slide and re-aspirating several times in succession. Mixture completed, an aliquot portion of the mixed fluids, such as suffices for our purpose, is drawn up into the capillary stem, and the orifice of the capillary tube is sealed in the flame. This done, the pipette is placed either in an incubator standing at 37° C. or in a vessel of water kept at this temperature.

After the lapse of fifteen minutes we break off the extremity of the pipette, carefully mix the contents so as to get an average sample, and proceed to make films, and then to stain them by Leishman's stain.

6. *The method of Wright and Douglas of determining the opsonic value of small quantities of blood* (*P.R.S.* loc. cit.).

Comparison is made between the number of bacteria ingested by living leucocytes when operating under the same general conditions, but in the serum of test, and in normal serum, respectively. The ratio between the figures yielded by the leucocytes in the two sera is the opsonic index. Thus, if the sample of leucocytes used ingest on the average eight bacteria

per leucocyte when acting in normal serum, and four when acting in serum from a given patient, then the apsonic index of this patient is $\frac{4}{8} = 0{\cdot}5$.

Three materials are required for arriving at the opsonic index.

(*a*) *Washed leucocytes.* The source of the corpuscles is immaterial, and the operator generally draws them from his own finger by pricking it and transferring the blood into a small test-tube filled with 1% sodium citrate dissolved in normal saline. After thoroughly shaking the mixture, the tube is centrifugalized, and when the corpuscles have settled to the bottom, the supernatant fluid is pipetted off and replaced by normal saline. The tube is then reshaken and again centrifugalized. The corpuscles may be washed in this way a third time, after which the tube is sloped in order to facilitate removal by pipette of the upper layer of the corpuscles, which contains the majority of the leucocytes.

(*b*) *Bacterial emulsion.* This is made in salt solution (0·9% as a rule, but 1·5% in case of the tubercle bacillus), and must be free of clumps. Bacteria should be neither too numerous nor too scanty in the emulsion.

(*c*) *Samples of serum.* Wright's blood capsule is the most convenient apparatus for collecting samples of blood for this purpose. The serum may be freed of corpuscles by centrifugalizing it. A good average sample of normal serum with which comparison is to be made may be obtained by mixing samples from three normal individuals.

It is convenient to have the washed corpuscles, bacterial suspension, and samples of serum in three

separate capillary pipettes, the ends of which are squarely cut by use of a glass-cutting knife.

The next step is to mix the corpuscles, bacterial suspension, and serum. A fresh pipette is therefore taken, and its lower capillary extremity cut across squarely by the glass knife. A mark is then made with ink or glass pencil about one inch or so from this extremity. The upper extremity of the mixing pipette may be either drawn out to a capillary and cut across, in which case it can be operated by the mouth; or a rubber teat may be fixed to it. The capillary pipettes containing the stocks of corpuscles, suspension, and serum are now taken in hand, and the lower end of each in turn slightly inclined. By holding the lower extremity of the mixing pipette in apposition with and slightly below the capillary extremity of each stock pipette, the fluid will readily run from one into the other – no suction is necessary —and the requisite volume of each can be taken into the mixing pipette up to the mark, a bubble of air being admitted after each volume. The proportions in which corpuscles, bacterial suspension, and serum were mixed by Wright and Douglas in their original research were as follows: —

Corpuscles	Suspension	Serum
3 vols.	1 vol.	3 vols.

but many are now content to take 1 vol. of each.

Having in this way obtained the required quantity of each of the three constituents in the mixing pipette the contents of the latter are expelled on to a sterile glass slide, watch-glass, or Petri dish, and thoroughly

mixed by drawing the fluid up into the pipette and expelling it a number of times. Air bubbles are broken by bringing a heated platinum wire near them. The fluid is then drawn up into the pipette, the ends of the latter sealed, the pipette labelled, the time noted, and the pipette placed in the 37° C. incubator. Fifteen minutes is the time usually allowed at this temperature to enable the leucocytes to engage in phagocytosis. At the end of the allotted time the pipette is removed from the incubator, its ends cut off with the glass knife, and its contents expelled on to a clean slide or watch-glass and again well mixed by alternately drawing the fluid up into the pipette and expelling it. A drop of the fluid is then deposited at one extremity of a clean slide, and by means of the square edge either of a cover-glass or of a slide, this drop of fluid is sharply but evenly drawn out over the slide. The drop should be of such a size that the film drawn from it ends at about the middle of the slide. The polynuclear leucocytes are mostly found at this far end of the film, for owing to their larger size they are dragged further than the red corpuscles. Wright has advocated rubbing the slides in their long axis with fine emery paper in order to groove them and thus facilitate separation of the leucocytes.

Each sample of serum is treated in exactly the same way in order that the degree of phagocytosis manifested therein may be comparable.

The films are next fixed and stained. In the case of ordinary bacteria, Leishman's modification of Romanowsky's stain is generally used (small tablets of this stain can be obtained: they are dissolved in

pure methyl alcohol). When the films have been stained and dried they can be examined directly with the oil immersion; but many prefer to place a little Canada balsam on a part of the film where leucocytes are numerous, to drop a cover-glass on the top, and to examine through that. The number of bacteria contained by at least 50 leucocytes may be counted. The average ingested in the patient's serum divided by the average ingested in the sample of normal serum gives the opsonic index of the patient.

When determining the opsonic index in regard to the tubercle bacillus a suspension of the dried bacilli can be used. Tubercle bacilli suitable for this purpose may be purchased and kept in stock. In this, as in all cases where the opsonic index is determined, it is absolutely necessary to obtain a suitable emulsion of the organism before proceeding with the test. The tubercle bacilli are ground up in an agate mortar in a little saline solution (salt 1·5%), and the emulsion freed of clumps by centrifuge or by filter-paper. The films are stained by the method of Ziehl Neelsen (p. 91).

7. *Leishman's method of determining the presence of 'stimulins'.* (*Trans. Path. Soc. of London*, vol. lvi, part 3, 1905. Also, Harrison, *Journ. of the R. A. M. Corps*, vol. vii, p. 329, 1906.)

Leishman has described the manner in which his phagocytic method serves to demonstrate the increased phagocytic activity induced in normal blood by the addition of a minute quantity of an immune serum. The substances in the immune serum upon

which this action appears to depend he found to be thermo-stable, specific and only present in immune bloods. Saturation experiments further showed that, when present, they could not be removed by the bacteria in question, as can be done in the case of opsonins.

Their presence may be demonstrated either by Leishman's original method or by Wright and Douglas's modification. In the former instance, a trace of the serum of an immunized person or animal —from $\frac{1}{25}$ to $\frac{1}{100}$ of the total volume of the mixture of blood and bacterial suspension—is added to the normal blood, the control specimen having a similar addition of a normal serum in order to make the results comparable. The phagocytic ratio is then ascertained in the usual way. When dealing with bacteria which are susceptible to the action of bacteriolytic substances in the immune serum it is well to heat the immune serum before adding it to the normal blood.

The modification of W. and D. may also be used. In this, the leucocytes of the blood are freed from the blood fluids by citration and a series of washings and centrifugings, the serum being finally restored at the moment of adding the bacterial suspension. The whole of the serum used in the estimation must be previously heated to 60° C. for 15 minutes in order to remove any thermolabile opsonin that may be present and, also, to obviate the under-estimation which may result in the case of a strongly bacteriolytic serum. The following mixtures are prepared :—

1. 'Control' tube.

Heated normal serum	3	volumes.
Bacterial suspension	1	,,
Washed blood cells	3	,,
Heated normal serum ($\frac{1}{5}$ dilution)	1	,,

2. 'Stimulin' tube.

Heated normal serum	3	volumes.
Bacterial suspension	1	,,
Washed blood cells	3	,,
Heated immune serum ($\frac{1}{5}$ dilution)	1	,,

The difference in the contents of the two tubes lies in the addition to tube 2, containing heated normal serum, of a trace of the heated immune serum, the possible effects of this dilution of the serum being controlled in tube 1 by a similar addition of diluted and heated normal serum.

Films are then prepared in the usual way after incubation of the tubes for 15 minutes at a temperature of 37° C.; these are stained, the ingested bacteria enumerated in the polynuclear leucocytes and the average calculated. The 'stimulin' effect is recorded by dividing the phagocytic index of the 'stimulin' tube (No. 2) by that of the 'control' tube (No. 1).

IX

Inoculation and Post-mortem Examination of Animals

Inoculation.

HAIR or feathers, if they interfere with the inoculation, are removed, the skin is cleansed with soap, followed by alcohol, and sterile water.

1. *Cutaneous inoculation.* Either the skin is slightly incised and a platinum loopful of the material inserted into the wound, or the skin is scratched and the material well rubbed in.

2. *Subcutaneous inoculation.*

a. In a pocket of skin. A fold of skin is raised up with the forceps, a small opening made in it with sterilized scissors, and a small pocket made in the subcutaneous tissue either with the point of the scissors or with a sterilized lancet. Into this pocket the material is introduced by means of a platinum loop.

Rats and mice are inoculated preferably at the root of the tail. They are grasped by the neck with dressing-forceps, and the tail is held in the hand. Guinea-pigs are inoculated on the chest or abdomen, rabbits in the ear, and birds in the breast.

b. With the syringe. Different kinds of syringe are used for subcutaneous injection. Among the best are those of Pravaz (with asbestos plunger), Koch, Stroschein, or Loeffler (with easily improvised rubber

plunger). The syringe must be sterilized before and after use, either in the steamer, or with alcohol and ether. Previous disinfection of the skin at the point of injection is unnecessary. The injection is made at the places mentioned above (guinea-pigs under the skin of the abdomen).

3. *Intraperitoneal injection.* The skin is incised and a cannula passed through the muscular wall into the peritoneal cavity.

4. *Intramuscular, or intrapleural injections* are made as above.

5. *Injection into the blood-stream.* Expose an easily accessible vein (i. e. the external jugular), introduce a finely-pointed cannula, and inject in the direction in which the blood is flowing (i. e. centripetally). Care must be taken to avoid introducing air, or death may result from air-embolism. In rabbits the ear vein is generally made use of for this purpose, and prior to injection pressure is exerted on the root of the ear to cause the vein to enlarge. If, when the injection is made, the surrounding tissue is distended, it is a sign that the fluid is not being injected into the vein.

6. *Injection into the stomach.* A plug of wood perforated by a hole is inserted between the front teeth, and through the hole in this plug a rubber catheter is passed into the stomach. The material is then poured down the catheter into the stomach. In the case of larger animals the material may be introduced into the stomach by enclosing it in pieces of potato. A cavity is made in a small piece of potato, filled with the material, and closed over with potato. The piece

of potato is then pressed back against the pharynx of the animal so that it is swallowed. Infected pieces of bread may be made use of in the same way.

7. *Infection by feeding.* When the infected material is not in itself nutritious it can be administered mixed with some food (e.g. a culture smeared or powdered on bread).

8. *Infection by the air passages.* This is effected by causing animals to inhale, in a tightly-closed inhalation apparatus, air contaminated with bacteria, or with infected dust. Or the material may be injected directly into the trachea.

Dosage of Inoculation Material.

Quantitative injections are effected in the following ways:—

1. *Fluid materials.* The quantity decided upon is measured out, dilution with sterile broth or with 0.8% NaCl solution being resorted to if necessary for this purpose, and the injection is then made with a graduated syringe.

2. *Solid materials.* For removal of the material, use may be made of a platinum loop at the end of a short platinum wire, the other extremity of which is fastened to a handle by a screw arrangement (like a certain form of pocket pencil). This detachable platinum loop is first stuck into a piece of cork and weighed, then charged with the inoculation material and reweighed. The difference between the two weighings gives the weight of the inoculation material attached. The contents of the loop are then distributed in a definite quantity of sterile fluid in such

manner that an easily measured quantity of the suspension (e.g. 0·5 c.c.) contains the quantity of the material which it is desired to inoculate, and after thorough shaking, this quantity is then injected. By using the same loop, the same inoculation material, and by filling the loop with as far as possible the same amount of it, equal results are obtained, and the weighing may be dispensed with. To this end a series of loops should be kept in stock of such size that they require about 2 mgr. of twenty-four hours' agar culture to completely fill them—so-called Normal Loops. Measurements for the preparation of loops of standard size have been given by Czaplewski.

Determination of the Number of Bacteria in the Dose of Material Inoculated.

The material is withdrawn from the culture as described above, and a quantity of the suspension equal to that used for injection is sown on agar or gelatine plates. If desirable, the suspension is first diluted with a measured quantity of sterile broth or 0·8% salt solution, and an aliquot part of this dilution sown. The colonies that develop are counted as in the case of plate cultures from samples of water (p. 220). In a similar manner the bacteria in all kinds of material may be estimated.

Preservation of Experimental Animals.

Mice and rats can be kept in labelled glass boxes or jars with wire covers, and guinea-pigs in stone pots with wire tops labelled with their weight, sex, and distinctive markings. Special diagrams of the outline

of these animals may be kept in stock for the purpose of recording their respective markings. Rabbits can be identified by staining their ears with aniline dyes. If preferred, numbered metal labels may be attached to the animals for the purpose of identifying them. Infected animals should be kept in separate cages, each when possible having a cage to itself.

Temperatures of infected animals are taken per anum by introducing a maximum thermometer with small bulb. Normal temperatures are as follows in Centigrade:—Dogs, 37·5–39·9°; Rabbits, 38·3–39·9°; Guinea-pigs, 37·3–39·5° (generally above 38°); Pigeons, 41·0–42·5°; Chickens, 41·0–42·5°.

Immunization of Animals.

This is effected by injecting increasing doses of toxin, or of living or killed bacteria, as the case may be, according to the kind of micro-organism used, and according to the object in view. One proceeds so that the first injection, be it either subcutaneous or intraperitoneal, &c., consists of a dose below the minimal lethal dose (M.L.D.) of the culture or toxin used. Any changes in the state of health of the animal are observed; and when it has completely recovered, particularly when its weight is not decreasing, one gives it a second injection consisting of a somewhat larger dose of the toxin or culture. Subsequent injections are carried out in the same way.

Immunization for the purpose of obtaining serum for Widal's reaction is effected by injecting cultures of typhoid, cholera, &c., that have been killed by exposing them to a temperature of 60–65° C. for upwards

of an hour. In the case of rabbits, a successful serum is as a rule obtained after 3-5 intravenous injections at intervals of seven days, the dose increasing from 1–10 loops of killed agar culture. Seven days after the last injection blood is withdrawn from a blood-vessel of the ear, or from the external jugular vein, or from the carotid artery. The vessel is exposed aseptically. Loose ligatures are placed above and below it, and the vessel then slit open in the direction of its long axis. After sufficient blood (which is received in a sterile vessel) has been withdrawn, the ligatures are tied. In the case of larger animals, without slitting the skin press the external jugular against the vertebral column and puncture the distal end of it with a trochar or syringe. For directions with regard to withdrawing blood from pigeons see p. 114. Stand the blood for 24–48 hours in the ice chest, and then draw up the serum in sterile pipettes (or centrifugalize).

Post-mortem Examination.

This should be made as soon after death as possible. If it has to be postponed, the cadaver should be kept fresh by preserving it in a cold room.

If possible, both before and during dissection, the animal, except in the case of large animals, should not be touched with the hands at all, but only with instruments. These instruments are prepared beforehand and kept ready. The animal is extended with its abdominal side uppermost and limbs stretched out from the trunk. The extremities of the limbs are secured to the dissecting board by pins or by loops of thread which are attached to hooks screwed into the

board. The skin of the abdomen and chest is now thoroughly moistened with sublimate solution in order to prevent the hair from interfering when the skin is divided. Or, before moistening with sublimate, the skin may be soaked and the hair shaved off; or the hair may be burnt off. In the case of birds the feathers are plucked from the breast and abdomen, and the skin then moistened with sublimate. The skin is now divided in the middle line from neck to symphysis pubis with sterile instruments, stripped on both sides to the junction of the limbs with the trunk, and pinned back. Burn the surface of exposed muscles where any hair may have fallen on them. Now with fresh sterile instruments a fold of the abdominal wall below the xiphoid cartilage is raised and divided, and with a knife the edge of which is turned upwards the abdominal wall is divided in the mid line, or, perhaps better, to the right of this line. The abdominal wall is then detached from the ribs on both sides, turned back and secured with pins to the flaps of skin previously pinned down. The flap which is pinned on the left side of the animal is made larger than on the right, in order that the spleen, which in most diseases is such an important organ, and is drawn out at a later stage, may rest upon it, and thus be protected from contamination by the skin. In rats and mice the muscles of the abdominal wall may be torn through with forceps instead of being divided with the knife. After the abdominal organs have been inspected, and the necessary cultures made therefrom, the thorax is opened by dividing the ribs on either side of the sternum with sterile scissors. The sternum is now

raised, and after the diaphragm has been detached from it, turned back so that the chest organs are exposed.

Whenever in course of the examination any instrument touches something likely to contaminate it, this instrument is at once changed or carefully flamed. *Never lay aside a used instrument without disinfecting or flaming it.*

Organs from which cultures are to be made are incised with a pointed sterile instrument, a platinum loop is inserted, and the material thus removed at once sown. If one has reason to suspect that an organ is contaminated, its surface is seared with a hot knife before proceeding as above. Should it be desired to sow larger pieces of the organ, one cuts it up with scissors and takes up the pieces with a hot platinum loop, to which they readily adhere. Hard nodules (e.g. in tubercular organs) may be excised with scissors, crushed between two sterile slides, and from the pieces thus separated cultures and cover-glass preparations can be made.

If it is desired to make the cultures at a later stage the pieces of organ may be removed aseptically and stored in sterile double capsules, each organ separately.

Organs which always contain bacteria, such as the intestines, should be opened last of all, and after cultures from the other organs have already been taken.

Always observe at the post-mortem the condition of the site of inoculation.

In making microscopical preparations one tears out

bits of tissue with the forceps, smears them on cover-glasses, and applies the stain to the films thus made.

For preservation of pieces of organ for sections see p. 60.

After completion of the examination the cadaver should be burnt in a furnace, enclosed with parchment-paper, or boiled in a steamer for 1–4 hours, according to the kind of micro-organism present. The board should be washed with 1–2% sublimate and 3% HCl. The pins, &c., flamed.

X

Methods of Preserving Preparations, Cultures, and Organs of Animals

a. Preparations.

Hanging-drop preparations. These can be preserved for a considerable time, but eventually further development occurs, and finally the micro-organisms disintegrate. If one wishes to preserve the preparation at a particular stage of development, one adds to the drop, or places on a corner of the cover-glass, a droplet of formalin or a trace of 2% osmic acid, and then replaces the cover-glass in its previous position on the slide.

Preservation of stained preparations, see pp. 59, 60.

Moulds and yeasts are preserved unstained—best in

glycerine-gelatine (glycerine 7, aqua 6, gelatine 1, carbolic acid 1%, warmed up together and filtered). Ring with varnish.

b. Cultures.

Cultures exhibiting characteristic growth (e.g. gelatine stab cultures of cholera showing typical air-bubble liquefaction) may be preserved. In that case further development is first arrested by exposing the culture to the action of formalin vapour.

When dealing with cultures in tubes, the under surface of the plug of wool is moistened with formalin and the plug replaced. The tube is then closed by a rubber cap and allowed to stand for at least twenty-four hours. If the culture has been made in gelatine and has liquefied it, the tube should be allowed to stand until the medium has become solid through action of the formaldehyde. After the culture has been exposed to formalin it may be treated in several different ways.

1. The tube may be preserved as it is. Conditions:—firm closure of the rubber cap; otherwise the culture gradually dries up.

2. The rubber cap is loosened, the plug of wool is pushed down in the tube, and a thick layer of melted paraffin is poured on the top of it and allowed to set. Should the paraffin subsequently fissure, the cracks must be filled up.

3. The rubber cap is removed, a round cover-glass which accurately closes the end of the test-tube is placed on the top, and a thick layer of glycerine-gelatine is painted over it (see above; instead of carbolic, 1% of sublimate may be used). When the glycerine-

gelatine has dried, it is covered over with a layer of varnish.

4. One heats a metal compound of low melting-point (e.g. Rose's metal, fusible metal, &c.) in a Bunsen burner, and allows a drop of it to fall from a height of $\frac{1}{4}$–$\frac{1}{2}$ meter on to a glass plate. The flat metal disk thus produced from the drop is applied to the mouth of the tube after removal of the rubber cap, its overlapping edge is pressed firmly down round the rim of the tube, and the latter is hermetically sealed by gently rotating it and at the same time warming it over the Bunsen burner.

5. The rubber cap and plug of wool are removed and the tube fused a short distance below its orifice. Take care that the medium is not impaired by the heat.

N.B.—Tubes which contain condensation water are always kept in a vertical position, or this water is carefully poured off before treatment with formalin.

Plate cultures may be preserved as follows:—

1. Two drops of formalin are placed on the inside of the cover, and the plate is allowed to stand for twenty-four hours. Whenever practicable, the plate should be inverted so that the formalin does not drop on to the medium; and a good method is to fasten a piece of blotting-paper on the cover of the plate, and to moisten this with the formalin. If at the end of twenty-four hours the formalin has not all evaporated, the remains of it are removed and a broad rubber band placed over the edge of the plate.

2. The culture is made on plates of which the cover is ground to fit and enclose the other half of the plate.

After growth has taken place the plate is then treated with formalin as in 1, and sealed off with melted paraffin at the point of contact.

3. The culture is made in a flat flask, shaped like a flattened test-tube, and dealt with as directed in the case of tube cultures.

Portions of agar and gelatine cultures can be preserved in the following ways :—

1. Treat with formaldehyde as directed above. Carefully excise the parts to be preserved, and transfer them to slides (or dry over H_2SO_4 till only a thin layer remains). Cover with glycerine and with a cover-glass, and ring with asphalt-varnish. The appearance of the colonies is impaired by this treatment, and the method is only applicable for agar plates.

2. Make a plate culture on a cover-glass, and after development has taken place, dry over H_2SO_4. Stain as in case of a dry preparation, and mount in Canada balsam. (Not suitable so much for inspection of colonies as for study of the arrangement of bacteria in them.)

c. Preservation of organs of animals for purpose of demonstration.

1. Place the organs in the following solution until completely decolorized:—Water 4,000, formalin 800, potassium acetate 85, potassium nitrate 45.

2. After allowing the fluid to drain off, place in 80% alcohol till the natural colour returns.

3. Preserve permanently in the following solution:—aq. dest. 900, glycerine 300, potassium acetate 200 (Strassmann's method).

XI

Bacteriological Examination of Water, Milk, Shellfish, Vegetables, Sewage and Sewage Effluents, and Soil and Excremental matters

(Revised and amplified by Dr. A. C. Houston, Director of Water Examinations, Metropolitan Water Board).

General Remarks. The usual object in making a bacteriological examination of water, milk, soil, &c., is to determine the quality thereof in relation to undesirable pollution. With this end in view, the full bacteriological examination is of threefold description; attention being directed towards ascertaining (1) the total number of bacteria present per gram or cubic centimetre of the material; (2) the relative abundance of certain definite bacteria significant of pollution; (3) the presence or absence of certain specific pathogenic bacteria. In all three cases the examination should invariably be quantitative.

Total number of bacteria and number of spores of bacteria. This is usually estimated by cultivating a known quantity of the material in gelatine plates at 20–22° C. The number of colonies is counted usually on the third day. The number of bacteria capable of developing at 37° C. can be determined by agar plates made in the same way, the colonies being

counted usually on the second day. If it is desired to estimate the number of spore-bearing bacteria present, a known quantity of the material is heated to 80° C. for ten minutes before being submitted to cultivation. The ratio between these three tests is often of importance. If a series of dilutions have been made, it is possible to form an estimate of the total number of micro-organisms present by inoculating tubes of fluid media (e.g. broth tubes) with falling quantities on the decimal scale, and by observing the dilution at which growth is no longer positive (e.g. 10,000, not 100,000 bacteria per c.c.; &c.).

Bacteria significant of pollution. Excremental material is the usual form of pollution for which substances of the above description are examined; and the index bacteria estimated are B. coli, streptococci, and spores of B. enteritidis sporogenes (Klein). It has been shown (L.G.B., 1902-3) that normal human excrement contains these bacteria to the following extent per gram:—B. coli (flaginac) about 100 million; streptococci at least 100,000; spores of B. enteritidis sporogenes about 1 million. It has also been found (second and fourth reports R.S.C.) that ordinary sewage contains these bacteria to the following extent per c.c. B. coli about 100,000 (over 75% of them flaginac); streptococci at least 1,000; spores of B. enteritidis sporogenes 100 to 1000. The foregoing figures are only approximate. By the relative abundance, therefore, of these index bacteria in a given sample, the amount of faecal contamination contained by it can approximately be measured. B. coli is usually estimated by inoculating the glucose

bile-salt medium of MacConkey with measured quantities of the material under examination. Streptococci can be estimated either by inoculating broth tubes in the same way and examining their growth microscopically (Gram-stained specimens) after incubation for 48 hours at 37° C., or by making surface cultures on plates of agar or Drigalski and Conradi's medium and subculturing the smallest colonies that develop. Spores of B. enteritidis sporogenes are usually estimated by inoculating milk in similar fashion and then incubating this milk at 37° C. anaerobically after subjecting it to a temperature of 75° C. for 10 minutes in a water bath. If the enteritidis change takes place in the milk, the result is recorded as positive.

It is necessary to isolate the Coli and streptococci in pure culture and to test their characters—the Coli in the flaginac tests, and the streptococci in the differential tests described above (p. 154). In the case of the enteritidis test the characteristic change in milk is sufficient for ordinary purposes; but if the case is a particularly important one, 1 c.c. of the whey should be injected into a guinea-pig and its pathogenicity tested.

Specific pathogenic bacteria. Special methods for isolating the typhoid bacillus and the cholera vibrio have been described elsewhere (pp. 137, 145). The presence or absence of anthrax, tetanus, or tubercle, can be determined by direct inoculation of animals with definite amounts of the material. Other pathogenic bacteria require special methods for their isolation.

Water.

Collection of sample. In the case of wells provided with pumps, samples are collected in sterile bottles after pumping for some time. In case of open wells, springs, streams, &c., the sample is collected in a sterilized bottle by hand if possible, or by lowering a weighted bottle with a cord; or it is collected by means of one of the special apparatus designed for this purpose (e. g. Esmarch's flask, which is closed by means of a weight covered with rubber, and can be opened and closed at any depth; or Sclavo's so-called 'smash bottles' consisting of a test-tube drawn out into a thin bent tube with fused extremity: when the tube has been sunk to the desired depth its drawn-out extremity is punctured by means of a weight which travels down a long cord, and the tube then rapidly fills with water).

Preservation of samples. In cases where it is impossible to examine the samples at once, they are packed in ice.

Enumeration of general bacterial content. The number of bacteria present per c.c. of the sample can be estimated by making gelatine plates from 1 c.c., 0·1 c.c., 0·01 c.c., and 0·001 c.c. of the water; the last three amounts being obtained by the dilution method.

If desirable, the number of bacteria capable of developing at 37° C. can also be estimated by making agar plates in the same way.

The Bacteriological examination of Water for Excremental Pollution.

1. The B. coli Test.

Stage 1. *Decimal mode of dilution.*

Add 9 c.c. of water to each of a number of test-tubes ($6'' \times \frac{3}{4}''$), plug and sterilize.

Label six of these tubes (1), (2), (3), (4), (5), (6).

(*a*) Add 1 c.c. of the water to be examined to tube 1 and shake.

(*b*) Add 1 c.c. from tube 1 to tube 2 and shake.

(*c*) ,, ,, 2 ,, 3 ,,

(*d*) ,, ,, 3 ,, 4 ,,

(*e*) ,, ,, 4 ,, 5 ,,

(*f*) ,, ,, 5 ,, 6 ,,

The values of the above dilutions may be expressed as follows:—

(*a*) 1 c.c. of dilution 1 = 0·1 c.c. of original water.

(*b*) ,, ,, 2 = 0·01 ,, ,,

(*c*) ,, ,, 3 = 0·001 ,, ,,

(*d*) ,, ,, 4 = 0·0001 ,, ,,

(*e*) ,, ,, 5 = 0·00001 ,, ,,

(*f*) ,, ,, 6 = 0·000001 ,, ,,

In the case of grossly impure waters it is necessary to make these six dilutions, but with potable waters it is seldom necessary to go beyond dilution (2). For purposes of description it will be assumed that only dilutions (1) and (2) have been made.

Stage 2. *The making of primary Cultures.*

Add 1 c.c. from dilution (2) to a test-tube ($6'' \times \frac{3}{4}''$ with an inverted inner tube $2'' \times \frac{1}{2}''$) containing

10–12 c.c. of single strength bile-salt glucose peptone water.

Deal similarly with dilution (1); then add 1 c.c. of the undiluted water to a third tube of the same medium.

Now add 10 c.c. of the water to a fourth tube containing 10–12 c.c. double strength bile-salt glucose peptone water.

Lastly, add 100 c.c. of the water to a fifth large tube ($8'' \times 1\frac{1}{2}''$ with inverted inner tube $3'' \times 1''$) containing 50 c.c. of treble strength bile-salt peptone water.

Stage 3. *The making of secondary Cultures.*

Withdraw a loopful from the primary tube and add it to a test-tube containing 10 c.c. of sterile water. After shaking, withdraw a loopful from the latter tube and smear it over the surface of an oblique, neutral red bile-salt agar tube, or a plate into which this medium has been poured. Incubate at 37–42° C. for 24 hours. B. coli shows as bright red colonies, and one or several of these colonies should next be sub-cultured into melted glucose gelatine and 'shake' cultures made. B. coli and like forms produce gas in this medium within 24 hours at 20° C. If gas formation in glucose is positive, the biological attributes of the microbe must be further studied.

Stage 4. *Determination of the type of B. coli.*

Many tests are available for this purpose, but a useful combination is the Flaginac test.

Four tubes containing different media are inoculated from the glucose gelatine culture, namely:—

Neutral red broth culture (fl = fluorescence within two days at 37° C.).

Lactose peptone culture (ag=acid and gas within two days at 37° C.).

Peptone water culture (in=indol within five days at 37° C.).

Litmus milk culture (ac=acid and clot within five days at 37° C.).

A micro-organism yielding positive results with all these four tests is described as a flaginac B. coli, that is, a microbe indistinguishable on the basis of the tests employed from typical B. coli of the alimentary tract.

Modifications.

Stage 2. Other media may be employed, e.g. ordinary broth, bile-salt lactose peptone water, phenolized broth, peptone water, &c.

Stage 3. Other media may be used, e.g. Drigalski and Conradi, ordinary agar, phenolized agar, ordinary gelatine, phenolized gelatine and various sugar agars.

Stages 3 and 4. A useful modification is the Quintuple Preferential B. coli test described in the Report on the Metropolitan Water Supply for January, 1907. Its description here would occupy too much space.

Stage 4. Additional tests may be used, for example: motility; Gram's stain; nitrate broth; growth on various solid media; reactions with various sugars and alcohols such as saccharose, raffinose, laevulose, mannose, dextrin, dulcit, isodulcit, mannite, salicin, &c.; Proskauer's and Capaldi media, &c. MacConkey has recently recommended Vosges and Proskauer's reaction and the following substances as having special differential value—lactose, saccharose, dulcit, adonit, and inulin.

It may here be added that in Stage 2, surface cultures on solid media may be used instead of liquid

cultures. But in this case it is usually necessary to concentrate the bacteria either by precipitation methods, or by filtration through a porcelain filter and subsequent brushing of the surface, or by centrifugalization, or by evaporation at blood heat under reduced pressure. Many solid media may be used for this purpose, e.g. gelatine and agar media containing various sugars, Drigalski and Conradi's medium, &c.

Eijkmann's test (*Centralbl. f. Bakt.*, Or. I. 37, p. 742) for the presence of B. coli in water depends on the fact that B. coli differs from most other organisms found in water in continuing to multiply at a temperature of 46° C. 100, 50 c.c. or less of the water are mixed with $\frac{1}{6}$ to $\frac{1}{8}$ its volume of concentrated peptone-grape sugar (see p. 23) solution in a large fermentation tube (see p. 48) and incubated at 46° C. Turbidity and gas formation indicate the presence of B. coli, which must be identified in the usual way. Should this test be negative, incubate some 50 c.c. of the water with broth for 24 hours at 37° C. and then add 1 c.c. of the culture to some of the peptone sugar solution (diluted ten times) and incubate in fermentation tubes at 46° C.

2. The Streptococcus Test.

For the purpose of dilution, stage 1 of the B. coli test may be used. But with moderately pure waters, it is desirable to search for streptococci in a large volume of water. Hence it becomes necessary to adopt one of two procedures :—

1. Concentrate the bacterial contents of the water either by precipitation, by porcelain filtration, by

centrifugalization, or by evaporation at blood heat under reduced pressure, and then make surface plate cultures on solid media. Many solid media may be used, but the medium of Drigalski and Conradi is well suited for this purpose.

2. Inoculate liquid media (e.g. broth with or without the addition of various sugars) with large volumes of the water and incubate at 37-42° C. for 24 hours Next make microscopic Gram-stained specimens of the liquid. If on microscopic examination streptococci are found present, surface plate cultures on Drigalski and Conradi's medium should be made.

Whichever procedure be adopted, it is necessary to study the type of streptococcus by microscopic examination and to investigate its biological attributes, including the reactions in the various differential tests described previously (p. 155).

3. The Spores of B. enteritidis sporogenes Test.

For the purpose of dilution, stage 1 of the B. coli test may be used. For larger volumes of water than 10 c.c., concentration of the bacterial contents of the water is necessary. The medium employed is 'whole' milk, and after inoculation with the water, the culture is heated to 80° C. for 10 minutes, and then incubated under anaerobic conditions at 37° C. After two days' incubation, the results are recorded; if the casein is precipitated and torn into irregular masses with a moderately clear whey and evolution of gas, the result is considered positive. But it is desirable to verify by animal inoculation and by various cultural tests.

There are many other tests which may be usefully employed in the bacteriological examination of water, e.g. determination of the ratio between the number of microbes growing respectively at 20° C. and at 37° C.; enumeration of the spores of aerobic bacteria; animal inoculation tests, &c. But these cannot be considered here. Similarly, special tests for specific pathogenic bacteria do not fall within the province of the present article.

The following media are used in connexion with the above method of examining water.

MacConkey's Bile-salt glucose peptone water.

Peptone 20 grams, glucose 5 grams, taurocholate of sodium 5 grams, 100 c.c. of a 10% sterile litmus solution, tap-water to 1 litre. Put peptone, glucose, taurocholate of sodium, and water in a flask, and heat in steamer for 45 minutes. Filter through Chardin's filter-paper, add the filtered litmus solution and place in tubes (with Durham's fermentation tubes). Steam for 45 minutes on two successive days.

Double strength bile-salt glucose medium.

The above solid constituents (peptone, glucose, taurocholate) are doubled. The medium is put into tubes in quantities of 10 c.c. The water to be examined is added to the medium in quantities of 10 c.c.

Triple strength bile-salt glucose medium.

The solid constituents are used in threefold strength. The medium is put into large tubes in quantities of 50 c.c. The water to be examined is added in quantities of 100 c.c.

MacConkey's Neutral red bile-salt agar.

Agar fibre 20 grams, peptone 20 grams, lactose 10 grams, taurocholate of sodium 5 grams, 4 c.c. of a 1% sterile aqueous neutral red solution, tap-water to 1 litre.

Soften agar in acidulated water as previously described. To the washed agar add peptone, bile-salt, and 500 c.c. tap-water. Heat in autoclave for 30 minutes, or in steamer for 90 minutes, then add remainder of water, cool to 60° C., add the beaten white of one egg, heat in autoclave for 45 minutes or in steam sterilizer for 90 minutes. Filter through a moistened Chardin's filter-paper, placing the funnel in a warm filter-jacket. When filtered, heat in steamer for 15 minutes, add lactose and neutral red, put in tubes, and steam for 30 minutes on two successive days. The medium requires no alkali.

Peptone litmus water

(used for sugar tests, with reference to B. coli).

Sugar 1%, peptone 20 grams, 100 c.c. of a 10% litmus solution, tap-water to 1 litre.

Milk.

For details as to the bacteriological examination of milk in relation to standards the reader is referred to a recent report (A. C. H.) to the L. C. C. (King & Son, 1905).

Sediment. On p. 27 of the report in question, a convenient method and apparatus are described and figured by means of which the volume of dirt deposited by a definite quantity of the milk can be

concentrated and measured, and known quantities of this deposit submitted to microscopical examination. The cover-glasses are 'flamed', immersed in ether for 5 minutes, transferred directly to absolute alcohol for a few seconds, and, without washing, placed film downwards in freshly prepared methylene blue solution (6 c.c. of the filtered saturated alcoholic solution of methylene blue, in 20 c.c. of a 1 in 10,000 solution of caustic potash) for not more than 10 seconds. After washing and drying, the preparation is mounted in Canada balsam in the ordinary way and is then examined with a one-sixth objective and a four ocular, the tube being drawn out to such an extent as to give a magnification of 460 diameters. Higher magnifications are necessary for special purposes. At least five 'fields' from each quarter of the cover-glass should be carefully examined.

Separate cover-glass preparations may also be stained as for tubercle, in the following manner: —

After treatment with ether and alcohol, the preparation is stained for several minutes in hot carbol-fuchsin, washed, decolorized in 33% nitric acid, washed and counter-stained with methylene blue. After washing, drying, and mounting, the preparation is examined for the presence of acid fast bacilli, with a one-tenth or one-twelfth oil immersion lens, in place of the one-sixth objective previously recommended. As already pointed out, failure to find acid fast bacilli affords no convincing evidence of their real absence. Moreover, not all acid fast bacteria are tubercle bacilli (see p. 101).

The cultural procedure used was as follows :—

Dilution. The decimal mode of dilution should be carried out as described under Stage 1 of the B. coli test under *Water* (p. 221).

B. coli Test.

Primary liquid cultures and secondary plate cultures method.—The bile-salt glucose peptone medium of MacConkey may be used for the primary cultures, and, for secondary 'plating' purposes, the solid media recommended under bacteriological examination of water for excremental pollution (see above). The procedure is as follows :—

Ten c.c. of the milk are added by means of a sterile 10 c.c. pipette to a tube containing 10 c.c. of double strength bile-salt glucose peptone medium.

A sterile 1 c.c. pipette is used for inoculation from the various dilutions in *reverse* order, i.e. 1 c.c. from dilutions (6), (5), (4), (3), (2), and (1), is successively added to a series of bile-salt glucose peptone tubes.

Finally, with the same pipette 1 c.c. of the undiluted milk is added to another bile-salt glucose peptone tube.

These cultures representing from 10 c.c. to one-millionth of a cubic centimetre of the milk are next incubated at 37° C. for two days.

All the primary cultures showing acid and gas formation are next subcultured in the way fully described under *Water*.

Primary plate culture method.—A gelatine medium or an agar medium (with or without the addition of one or another sugar) may be employed. But there is convenience in using the Drigalski and Conradi medium as this is specially suited for the isolation

of streptococci, and also of the non-lactose-fermenting group of B. coli.

The procedure in any case is the same. The plates are inoculated with 0·1 c.c. of the various dilutions, and the material spread over the surface of the plates by means of a suitable sterile instrument. The plates are incubated at 37° C. and after 24–48 hours carefully examined. Selected colonies are subcultured in various media in the manner already described.

Streptococcus test.

As previously explained, the Drigalski and Conradi medium is well adapted for the purpose in view. The plates are inoculated with 0·1 c.c. of the various dilutions and the material spread over the surface of the plates by means of a suitable sterile instrument. After incubation for 24–48 hours at 37° C. the minute colonies are subcultured in broth. The broth cultures are incubated for 24–48 hours at 37° C. and then examined microscopically. If the results of the microscopic examination prove satisfactory, the streptococcus is subjected to the differential tests for streptococci described above (p. 155) and also to the nitrate broth test (for observation as regards *absence* of ability to reduce nitrate to nitrite).

An alternative method is to inoculate a series of lactose peptone (+ Lemco) tubes from the various dilutions. These tubes are incubated at 37° C. for 24–48 hours. As the great majority of milk streptococci produce acid in a lactose medium, only those tubes which show an acid change need be studied. Microscopic preparations from the tubes showing

development of acidity are next made to see whether streptococci, mixed or unmixed with other bacteria, are present. If streptococci are present, secondary plate cultures must be made and the microbes studied in pure culture. This method works fairly well if the streptococci are present in large numbers compared with other microbes. But under these conditions almost any method would yield satisfactory results.

B. enteritidis sporogenes test.

The following amounts of milk should be used for cultural purposes:—100 c.c.; 10 c c.; 1 c.c.; ·1 c.c.; ·01 c.c.; ·001 c.c. The 100 c.c. of milk is added to a large sterile tube; the 10 c.c. of milk to a large tube containing 30 c.c. of sterile milk, and the rest of the amounts to ordinary tubes containing 15 c.c. of sterile milk. The cultures are heated after inoculation, to 80° C. for ten minutes for the lesser amounts, and 15–20 minutes for the larger amounts of milk. The cultures are incubated, under anaerobic conditions, at 37° C. and observations made as regards the 'enteritidis change' after two days' incubation.

Shellfish.

The following method of examining oysters bacteriologically is taken from a Report (A.C.H.) to the Royal Commission on Sewage Disposal (Fourth Report, vol. 3; also *Journal of Hygiene*, vol. 4, no. 2, p. 185). The directions given apply, with modifications, to the examination of shellfish in general.

Cleansing of the Oysters:—

The outside of the oyster shell is well scrubbed with soap and water, and cleansed as thoroughly as

possible under clean running water; the shells are then well washed in running *main* water, and finally with sterile water.

Cleansing of the Hands:—

The hands of the experimenter are thoroughly cleansed with a hard scrubbing brush, soap and water, then rinsed first with 1 in 1,000 corrosive sublimate solution and finally with sterile water.

Subsequent procedure:—

The oysters are laid out upon a sterile towel, the flat shell uppermost. They are opened in this position with a sterile knife, held in the right hand, while they are held in position with a corner of the sterile cloth grasped in the left hand. Great care is taken to avoid any loss of the liquor. This liquor in the shell is poured into a sterile 1,000 c.c. cylinder, the oyster is then partly cut up with sterile scissors, and the liquor thus freed also allowed to run into the cylinder; finally, the oyster is cut up into small pieces and added to the cylinder. Ten oysters are thus treated in each experiment. The volume of oyster + oyster liquor is read off, and usually varies between 80 and 120 c.c., so that the oysters, being of medium size and containing a medium amount of liquor, 100 c.c., might be considered a fair average of the total shell contents of ten oysters. Sterile water is then poured into the cylinder up to the 1,000 c.c. mark, and the whole well stirred with a sterile rod.

The following amounts of this liquid are taken for cultural purposes (primary cultures):—

Culture A 100 c.c. = contents of 1 oyster
„ B 10 c.c. = „ „ $\frac{1}{10}$ „
„ C 1 c.c. = „ „ $\frac{1}{100}$ „

Culture (1) 1 c.c. of $\frac{1}{10}$ dilution = $\frac{1}{1000}$ oyster
„ (2) 1 „ $\frac{1}{100}$ „ = $\frac{1}{10000}$ „
„ (3) 1 „ $\frac{1}{1000}$ „ = $\frac{1}{100000}$ „
„ (4) 1 „ $\frac{1}{10000}$ „ = $\frac{1}{1000000}$ „

These amounts—A, B, C, (1), (2), (3), (4)—are used for examination for B. coli (primary cultures), and the amounts B. C. 1, 2, 3 for the B. enteritidis sporogenes test. Experience has shown that it is best to make the primary cultures in triplicate. Then if, as regards the B. coli test, a sugar medium is employed, at least two out of the three primary cultures should form acid and gas to allow of a preliminary numerical diagnosis being made. Similarly, in respect of the B. enteritidis sporogenes test, the 'enteritidis' change should occur in at least two out of the three anaerobic milk cultures to merit a positive result being recorded.

The subsequent procedure, so far as the B. coli test is concerned, is the isolation of B. coli or coli-like microbes from the primary cultures, by means of secondary bile-salt agar cultures followed by study of the isolated microbe in the pure state. The chief advantages of this method are as follows:—

1. It is a *definite quantitative* method, succeeded by qualitative records.

2. It gives the average volume of the whole contents of the oyster shell.

3. It yields results based on collective examination of ten oysters.

4. It includes the examination of the entire contents of the shell.

5. The results can be stated as number of bacteria, either per oyster, or per c.c. of oyster.

Watercress or similar Vegetable material.

The following method of examining watercress is taken from a report (A. C. H.) to the London County Council (King & Son, 1905). If necessary, lettuce or other similar material can be examined in the same manner.

The bacteriological examination of the first and final 'washings' of the cress.

One hundred grams of watercress are weighed out and transferred bit by bit by means of a sterilized forceps and scissors to a flask containing 900 c.c. of sterile water. The flask is vigorously shaken for some time and then cultures are made of the 'washings' of the cress as follows :—

(*a*) One hundred c.c. are transferred into a flask containing 100 c.c. double-strength bile-salt glucose peptone medium. (This culture represents the first 'washings' of 10 grams of the cress.)

(*b*) Ten c.c. are transferred into a tube containing 10 c.c. of double-strength bile-salt glucose peptone medium. (This culture represents the first 'washings' of 1 gram of the cress.)

(*c*) One c.c. is added to a tube containing 10 c.c. single-strength bile-salt glucose peptone medium. (This culture represents the first 'washings' of 0·1 gram of cress.)

Dilutions are made as follows:—

Ten c.c. of the washings are added to 90 c.c. of sterile water in a flask called (2).

Ten c.c. of (2) are added to a second flask containing 90 c.c. of sterile water called (3).

Ten c.c. of (3) are similarly added to a third flask called (4), 10 c.c. of (4) to (5), and lastly, 10 c.c. of (5) to a fifth flask called (6).

One c.c. from flasks (2), (3), (4), (5), and (6) are added to tubes containing bile-salt glucose peptone, and they represent respectively the first 'washings' of ·01, ·001, ·0001, ·00001, and ·000001 grams of cress.

The foregoing cultures are called primary cultures. They are incubated for 48 hours at 37° C.

The primary cultures which, after incubation for 48 hours at 37° C., fail to develop 'gas' are discarded, because this negative result conclusively proves the absence from such cultures of glucose-fermenting coli-like microbes, of any kind. On the other hand, the occurrence of gas in the primary cultures constitutes the *prima facie* or presumptive test for B. coli, and indicates the necessity for making from the liquid secondary cultures on solid media. As regards these secondary cultures reference should be made to stage 3 of the bacteriological examination of water for excremental pollution.

Reverting to the stage of the process at which the primary cultures representing the first 'washings' of the mixture of cress and water have been made: The next step is to pour off the remaining water in the flask, to add fresh clean water, shake the flask, then pour off the water, again to add fresh water and again

to pour off the water. Repeat this procedure several times; then add to the flask sterile water, shake the flask and pour off the water. This should be repeated several times. Finally, add 900 c.c. of sterile water to the flask, shake it repeatedly, and proceed on exactly the same lines as before to examine the liquid (called the final 'washings'). During the process of washing, any simple device, such as holding a piece of sterile wire gauze over the mouth of the flask will prevent loss of small floating pieces of cress.

As the final 'washings' liquid is examined in exactly the same way as the first washings, it is unnecessary to repeat what has been already described.

Mention has only been made so far of the B. coli test, but of course the first and final 'washings' of the cress may be examined by the streptococcus, B. enteritidis sporogenes, or other tests.

Known quantities of the cress may also be added directly to primary liquid cultures, the subsequent procedure being on the lines already indicated.

Sewage and Sewage Effluents.

The method by which these materials are examined bacteriologically is similar to that practised in the case of water, but in the present instance dilution is carried further.

Detection of specific pathogenic bacteria in sewage is not easy by cultural methods at present available, and so far the animal experiment would appear to have afforded the best positive results. By this method Houston succeeded in demonstrating anthrax in the coke beds and septic tank sludge at the sewage works of a town where a number of tanneries are

situate; and he further showed the presence of this same specific pathogenic micro-organism in mud of a stream contaminated by sewage from the same town (*Second Report*, R.S.C.). Among other pathogenic micro-organisms that have been recovered from sewage and sewage effluents are B. pseudo-tuberculosis and B. pyocyaneus.

Soil.

A platinum or metal spoon may be used for collecting a sample of the soil to be examined.

When examining the deeper layers of soil, either a hole is dug and samples taken from its sides at the required depth and inoculated, or Fraenkel's borer is used. The latter is driven into the earth by rotating it in a certain direction, and when the desired layer is reached, it is twisted round several times in the opposite direction, whereby a chamber in the point of the borer is opened and filled with earth. Then one draws the instrument out by rotating it in the first direction.

Examination of soil for excremental pollution. A definite quantity of the soil (e.g. a gram or more) is ground as finely as possible by means of a sterile pestle and mortar, or else is shaken up in a flask with sterile shot, &c., and is then diluted with known quantities of sterile water: the decimal mode of dilution being preferable. Cultures are then made from definite quantities of these dilutions, and the number of B. coli, streptococci, spores of B. enteritidis sporogenes, determined per gram of the original material. The characters of the B. coli and strepto-

cocci isolated should be determined in detail by the tests described elsewhere. For further details as to the bacteriological examination of soil for excremental pollution, see Reports (A. C. H.) to the Local Government Board, 1899-1900, ib. 1900-1, ib. 1901-2, also Savage, *Journal of Sanitary Institute*, 1903.

Specific pathogenic bacteria. When examining samples of soil for infective micro-organisms (tetanus, malignant oedema, anthrax, tubercle), one injects animals subcutaneously, either with some of the material itself or with some 0·8% NaCl solution with which the material has been shaken up.

Excremental Matters

(e.g. faeces, cow dung, &c.).

The reader is referred to reports (A. C. H.) to the Local Government Board on this subject. But practically the same methods may be used as those described for liquids, except that it is necessary before making the various dilutions to thoroughly mix a known weight of the solid with a known volume of water.

XII

The Bacteriological Examination of Dust and Air

(Revised and amplified by Dr. Gordon).

Dust.

If in sufficient quantity, the dust may be collected with a sterile knife and transferred to the laboratory in a sterile test-tube. Dust may also be collected from the air by exposing a sterile glass box, a sterile enamelled tray, or, if within doors and under a grating, a piece of highly glazed paper.

Examination for excremental pollution. A gram, or if possible, more, of the dust is weighed out, well crushed up in a sterile mortar with a little sterile water, and diluted up to a bulk of 100 c.c. for the primary dilution. From this a series of decimal dilutions are made through successive flasks as described above for milk. A series of cultures are then made in gelatine (22° C.) or agar (37° C.) for total numbers; in MacConkey's fluid for B. coli; in broth or on Drigalski and Conradi's medium for streptococci; and in milk for the spores of B. enteritidis sporogenes; the last cultures being heated to 75° C. for 10 minutes before incubation anaerobically

at 37° C. The B. coli and streptococci are isolated in pure culture and their characters examined.

The result of some analyses of dust made on the above principle are seen in this table. The figures refer to the number approximately per gram of dust.

Source.	No. of Bacteria (37° C.).	B. coli.	Strepto-cocci.	B. enteritidis sporogenes.
Window bars and stonework at air inlet, House of Commons.	under 10	under 10	under 10	under 10
Week's sample of dust arrested from incoming air by scrim-cloth screen.	10,000	100	under 100	1000
3-months' sample of material falling through gratings in floor of Debating Chamber from boots.	100,000	100	10,000	1000
Dust of Debating Chamber.	100,000	1000	10	1000
Dust of Division Lobbies.	1,000,000	1000	1000	1000
Dust of New Palace Yard (cabs present).	100,000	10,000	under 1000	100

Air.

The following methods enable an estimate to be formed of the number of bacteria contained by the air.

1. *Hesse's method.* A glass tube 70 cm. long and 5 cm. wide is used. Gelatine is either spread all over the interior in the manner of a roll culture, or when, as is generally the case, the tube is used in a horizontal position, it is sufficient if a layer of gelatine 5 mm. deep is solidified on the floor of the tube throughout its whole length. The inlet end of the tube is provided with a rubber cap pierced by a hole, and to the opposite end of the tube an aspirator is attached by means of which air is slowly (10 litres in twenty minutes) drawn through. Tube, rubber cap, and gelatine are previously sterilized. Even in case of air comparatively free of micro-organisms it is advisable not to draw through more than 100–200 litres, otherwise the colonies lie too closely together. In the end of the tube to which the aspirator is attached (outlet end) a plug of sterile wool may be placed, and when the air has been drawn through, this plug may be sown in a gelatine plate. If the air has been drawn through sufficiently slowly, this plug remains free of bacteria.

2. *Petri's method* enables the micro-organisms to be collected from a larger quantity of air. By means of a pump with automatic counter attached, a measured quantity of air is drawn through two sand-filters, which are contained within the same glass tube and are separated from each other and from the outside

by small disks of metal gauze. The whole is of course sterilized before use. After the air has been drawn through, the sand and gauze are sown in gelatine or agar plates. If the filters are firmly pressed together, all the micro-organisms are held up in the first one. Instead of sand, pulverized sugar may be employed (it is, however, difficult to sterilize). Also grains of glass measuring from 0·25 to 0·5 mm., and contained in tubes of particular construction, may be used as a filter (Ficker, *Zeit. f. Hyg.*, Bd. 23).

3. The following method of *Bujwid* has the merit of simplicity. Air is slowly drawn through three wash-bottles communicating with each other, with rubber stoppers, and glass tubes, and each containing 2–3 c.c. of sterile water. The air yields up its micro-organisms to the water, various quantities of which are at the end worked up with gelatine.

4. *Frankland's method* (*Phil. Trans.* 1887, No. 2, p. 127). A known volume of air is drawn through a short piece of glass tubing constricted at two places to support plugs made of powdered glass wool mixed with sugar. The first plug holds up the micro-organisms, and cultures made from the second should remain sterile. This method has superseded Hesse's method.

5. *Method of Straus and Wurtz* (*A. I. P.* 1888, p. 171). A measured quantity of air is drawn through a special piece of glass apparatus containing 10 c.c. of sterile melted gelatine. The fluid retains the micro-organisms, and cultures are made subsequently from measured amounts of it. A drop of sterile oil is placed on the top of the fluid to control bubbling.

Broth may be used in place of gelatine, if desired. Air may be drawn through at the rate of 50 litres in fifteen minutes. The method is asserted to be more accurate than the others mentioned above.

Examination of Air for the Presence of Particulate Contamination.

The air of inhabited rooms is specially liable to derive particulate pollution from the presence of droplets of mucus sprayed from the mouth in the act of sneezing, coughing, and loud speaking (Flügge); from particles of epidermis shed from the surface of the body; and from dirt brought in from the streets on boots of the occupants. Certain bacteria, possessed of constant and well-defined characters, furnish means whereby pollution of each of these three kinds can be detected.

(1) *Pollution from material derived from the upper respiratory passages.* It has been shown (Gordon, *L.G.B.* 1902–3, ib. 1903–4) that certain streptococci, owing to their constant abundance in normal human saliva, provide a test whereby the presence of particles of that material may be identified, and that Streptococcus salivarius (see p. 155) in particular furnishes a means whereby 0·000,001 c.c. or less of saliva can be detected in air.

(2) *Pollution from material detached from the skin.* Owing to its constant abundance on the normal skin, Staphylococcus epidermidis albus forms a means whereby particles detached from the surface of the body may be detected (Gordon, *L.G.B.* 1904–5). The

characters of this staphylococcus have been stated before (p. 156).

(3) *Pollution by material brought in from the street on boots.* Material of this kind consists largely of horse-dung, and may be recognized by the presence of B. coli, spores of B. enteritidis sporogenes, and certain streptococci. B. mycoides is also not infrequently contained in such material, and thus serves as an additional bacterial index whereby its presence may be detected in air.

Particulate pollution of the three kinds mentioned above is most readily recognized by exposing plates filled with broth to the air, by then incubating this broth anaerobically for 48 hours at 37° C., and isolating and testing such index micro-organisms as may have developed in the broth by that time. Plates of solid media may also be exposed for the same purpose, and to this end solidified blood serum, ordinary agar, or the medium of Drigalski and Conradi may be exposed to the air.

Sewer Air.

Recent observations made by Andrewes and Hurtley (*Report on the sewer air of Hampstead*, 1905), and by Horrocks (*P.R.S.*, Feb. 7, 1907), show that by similar methods particles of sewage may under certain circumstances be detected in the air of sewers or drains. Horrocks has even succeeded by this means in demonstrating the presence of B. typhosus in the air of a drain through which typhoid excreta were passing (loc. cit.).

INDEX

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA

MEMORANDA