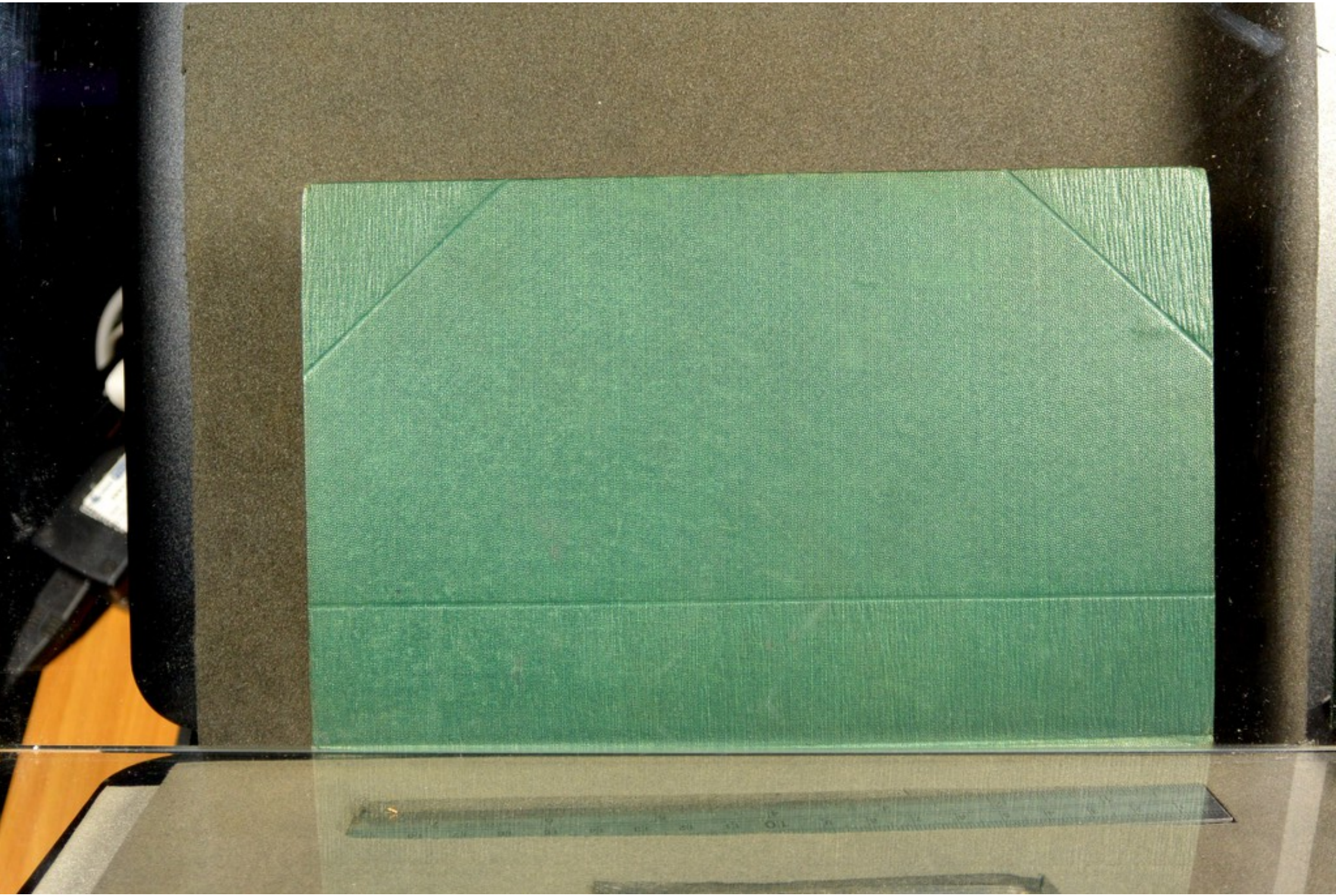
Studies in laboratory work / by C.W. Daniels.



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BY

C. W. DANIELS, M.

Late Medical Superintendent of the Londo
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1913

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LABORATORY WORK

BY

C. W. DANIELS, M.B., M.R.C.S.

Late Medical Superintendent of the London School of Tropical Medicine;
Director of the Institute for Medical Research,
Federated Malay States.

London
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PREFACE.

THE object of this work is to assist practitioners in the Tropics in the application of simple laboratory methods to the practice of medicine.

The writer has had personal experience in several countries of the peculiar difficulties that a student desirous of advancing the knowledge of tropical medicine, or of practising it conscientiously, will meet, and the plan of study advocated is the outcome of this experience.

The subjects include an outline of animal parasitology and the development of the best-known of these parasites. The part played by insects in spreading disease is so important that it is necessary to have a sound working knowledge of the more important known carriers of disease.

Chapter vii. has been kindly written by Mr. F. V. Theobald for this book, so as to enable the student to differentiate the more important genera of the *Diptera*. No exhaustive study of any one subject has been made, but it is hoped that sufficient information is given to enable the practitioner to commence the effective study from the laboratory point of view of the more important problems. Simple methods are selected as far as possible, and those recommended are in the main those adopted by the writer for teaching purposes at the London School of Tropical Medicine, and can be relied on as applicable to the circumstances.

Few references are given, as the practitioner in the Tropics has rarely access to a library.

I am much indebted to Dr. G. C. Low, Medical Superintendent at the London School of Tropical Medicine, for valuable assistance, and the revision of the proofs has also been kindly undertaken by him.

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CONTENTS.

LIST OF ILLUSTRATIONS.

ERRATA.

Page 50, Fig. 20 upside down.
„ 63 last line, for "inch" *read* "plate."
„ 132, Fig. 46, Spectra are inverted whilst the numerals and letters are not. The numbers of the Spectra and the key is omitted.
„ 134, line 1, after "resistance" for "of" *read* "or."
„ 192, in (*a*) (2) insert "narrow curved" between "No" and "head."
Figs. 93, 94, 95 and 96. *read* "after Nuttall."
Fig. 102 *d*, for "Panoplites" *read* "Mansonia."
Page 248, 3rd para., for "Microscopic" *read* "Macroscopic."
„ 250, line 11, for "alcoholic" *read* "acholic."
„ 252, line 30, for "the" before eggs *read* "other."
„ 254, line 28, for "if" *read* "it is."
„ 308, line 7, for "forming" *read* "fuming."
„ 309, last line but one, for "disease" *read* "diseases."
„ 345, Chart I., for "Percentage of bodies having Enlarged Spleens" *read* "Percentage harbouring parasites of Malaria."
For "J. H. Wright" *read* "A. E. Wright."

Studies in Labor

CHAPTER

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Studies in Laboratory Work.

CHAPTER I.

THE LABORATORY.—In few places in the Tropics will there be any institution that will correspond with the British idea of a laboratory. Tap water, gas and electric light have usually to be dispensed with and substitutes employed. The isolated worker has to arrange and make his own laboratory, either in the house or attached to a hospital. A separate building will rarely be available.

The first essential is a good light, and if, as is usual, work is done by daylight, the light must come neither from east or west. A north or south aspect should be chosen, according to the position whether north or south of the line, so as to avoid direct sunlight.

A corner of a verandah makes a good laboratory, with blinds or jalousies on two sides, and only the one side, that facing north or south, open. The side from which the light is received should be closed in with a window if possible, to prevent rain and dust entering.

Another important consideration is wind, and with the wind the amount of dust. If there is a glass window this is of less importance, but if working on an open verandah, a portion of the verandah must be selected sheltered from the prevailing wind, even if a south aspect has to be used instead of a better northern one, or *vice versâ*.

On the wall should be fixed a number of plain wooden

shelves. One of the lower ones at a convenient height should be strong and broad enough to receive a heavy weight. On this shelf may be kept mosquito cages, maturing larvæ and other objects awaiting immediate examination or requiring constant attention.

Two tables are better than one. It is convenient to have one table on which to work with the microscope, and also to keep papers, notebooks and any book actually in use; whilst on the second table staining processes, dissections and the rougher and more messy work can be done. Individual habits of neatness and arrangement make a difference. Though much excellent work has been done by untidy workers, there is no doubt that in the limited space necessitated by the narrow verandahs usual in many parts of the Tropics, work is easier and more comfortable if the habit of tidiness be cultivated. Persons exceptionally neat and methodical in their habits will probably find one long table more convenient than the two recommended here.

A firm, steady table should be placed a few feet from the window for work with the microscope. The second table, also strong, must be placed in a good light, and it is better to have the light falling from the left-hand side of the table.

Jalousies are most convenient in a hot place for the other sides of the laboratory, as they let in plenty of air and can be turned so as to regulate the amount of air and to stop the rain.

A cheaper arrangement is to use reeds. Natives in most parts of the world are good workers with reeds, and sufficient air will pass through to keep a room cool. Native mats can be used, but must be nailed on to a framework, as otherwise they will get blown about by the wind.

Water must be kept in bulk as tap water is rarely available. A small tank—an empty thoroughly cleaned

kerosine tin will serve—should be kept filled with water. This should be filtered, and a glass syphon tube with a rubber tube and clamp attached can be used to draw it off. The tank must be kept covered with a well-

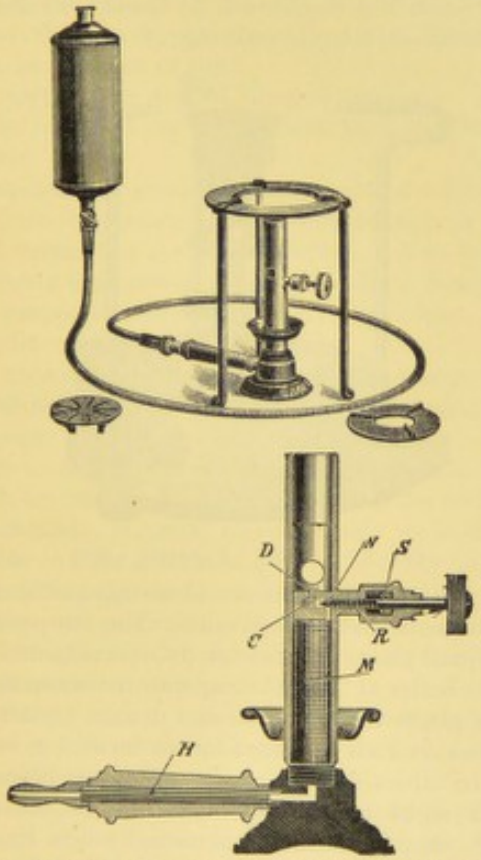

FIG. 1.—AUTOMATIC BUNSEN BURNER FOR METHYLATED SPIRIT.

fitting lid, and a basin or other receptacle to receive the waste and washing of stains, &c., should be placed underneath.

Distilled water must be kept in bulk in a well-stop-

pered bottle, from which a sufficient amount is taken as required into a wash bottle for immediate use.

An excellent substitute for the ordinary gas Bunsen burner is the spirit Bunsen (fig. 1). The "Primus" Kerosine Smokeless Burner will be found very useful for heating vessels on a larger scale (fig. 2).

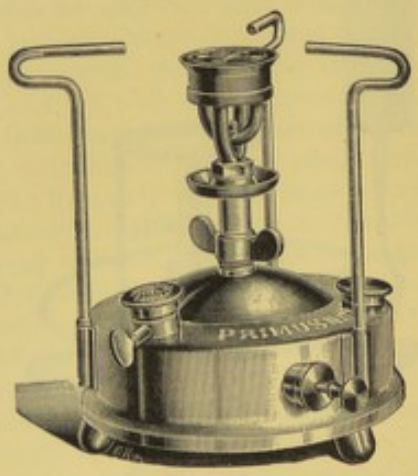

FIG. 2.—"PRIMUS" PARAFFIN LAMP.

An incubator is an enormous advantage and is essential for accurate bacteriological work. The temperature in most tropical places ranges from 75° upwards, and organisms grow better at "room" temperature than in England. In many places the nocturnal and diurnal variations are small, and in such the need for an incubator is not so great. In others there is a great difference between the day and night temperature and in these the need is greater.* A cold incubator is useless unless ice can be obtained.

* With practice and the exercise of some ingenuity a workable incubator can be made by placing one tin inside a larger one; (or a chemist's water-oven may be employed). The space

Cultures should be kept in a dark cupboard as dry as possible.

Above the long broad shelf running along the wall two or three rows of narrow shelves should be fitted up on which stains in use can be kept. These are better kept exposed than in cupboards. The main stock can, of course, be kept out of sight.

For night work a good lamp is required. The lamp must be low, and not raised more than six inches from the table.

Equipment : A good microscope with a sub-stage condenser, iris diaphragm and mechanical stage is essential. An oil immersion $\frac{1}{12}$-inch objective, a low, say $\frac{2}{3}$-inch, and a fairly high power, say $\frac{1}{6}$-inch, will be required. For many purposes a $\frac{1}{4}$-inch is a very useful lens, and it is well to have two eye-pieces.

The choice of suitable microscopes is a large one, and the differences between those of different makers are not very great, the points of difference being such that it is difficult to say which is the best. In the choice much depends on the conditions under which the work has to be conducted. If much travelling has to be done it is advisable to have a microscope that is easily carried and can be set up for use at a moment's notice. Of these travelling, or portable, microscopes there are several different forms all fulfilling the main requirements—lightness, compactness, and usefulness.

The folding microscopes made by some makers, though compact and therefore easily packed, are heavy and therefore inconvenient to carry.

If most of the work can be done at a fixed station one

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between the two is filled with water. A small kerosine lamp placed below the tins will heat the water, and by varying the height of the lamp sufficiently equable temperature can be maintained.

of the ordinary forms of microscope is the best, as they are the most convenient to work with. If the expense is no object it is well to have two stands, one portable

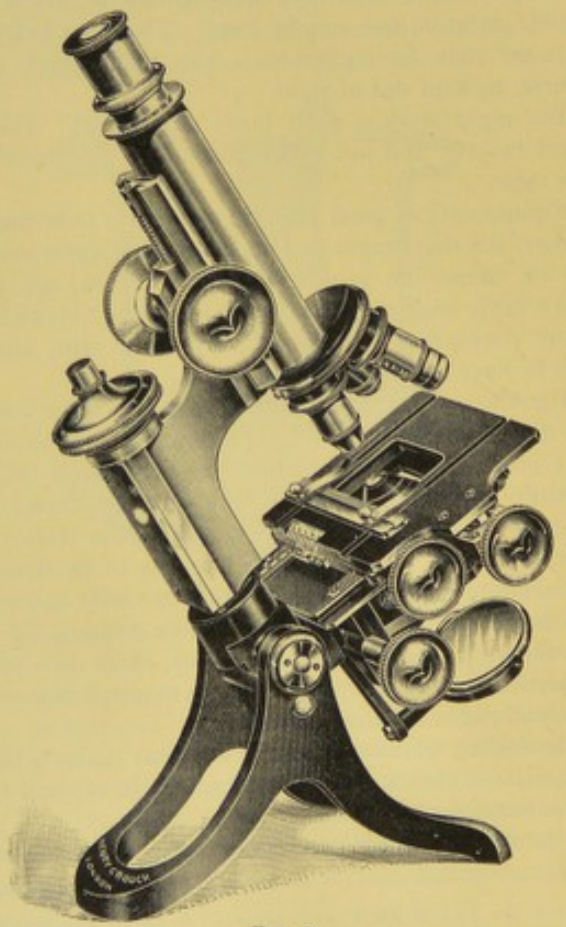


FIG. 3.

and one for stationary work. The objectives and eye-pieces can be used for either, and therefore the additional expense is not very great.

PARTS OF A MICROSCOPE.—The base or stand is either a stage fixed on to a tripod or to a vertical column rigidly attached to a solid and heavy footplate. The tripod is to be preferred, as from the wide spread of the legs greater stability is insured, and the level is less affected by irregularities in the table on which the microscope is placed.

In the folding and portable microscope the legs of the tripod are jointed at or near the junction with the stage and can be folded back so as to economise space in packing.

The stage itself is a fixed plate firmly attached to the upright carrying the optical parts of the instrument, viz., the mirror and sub-stage condenser below the stage and the tube, eye-piece and objective above.

To this solid plate is affixed the mechanical stage, of which there are two main types :—

(1) Those in which a lighter stage carrying the object to be examined is attached above the fixed stage. This can be moved by a rack and pinion in two directions at right angles to each other.

(2) In the second class the slide is seized by catches and moved over the solid stage.

Some mechanical stages have in addition to the rectangular motions a circular one in the same place. This motion is not required.

Of the two types preference should be given to the first, as it can be used for objects of all sizes and shapes, not simply, as with the second, for objects mounted on the regulation slides. With care it does not get out of order any more readily than that of the second type.

The microscope tube is attached to the upright in such a manner that it can be moved up and down parallel to the upright, but allows no lateral movement in any direction. The length of the tube is important, as with the higher objectives the best definition is obtained with a

certain known length of tube. This distance varies with the objectives of different makers. To provide for this variation there is a second or draw-tube inside the outer tube which can be drawn out so as to lengthen the tube to the required extent. The length of tube required for an objective should be ascertained and the draw-tube should be, and usually is, marked so that the corresponding length can be obtained.

In the portable and folding microscope the outer tube is so short that it is always necessary to use the draw-tube.

The adjustments by which the object is focussed are of two kinds :—

(1) The coarse adjustment, by which the tube is moved by a rack and pinion and brought approximately into focus. The range of the coarse adjustment is great, but the movement is too coarse to focus easily and correctly with higher powers.

(2) The fine adjustment, which may be a differential screw or of the lever pattern. The range of this adjustment is small, but very delicate movement is obtained.

Illuminating Apparatus.—Good illumination is absolutely necessary for useful work with high powers. The parts of the microscope providing for this illumination and modifying it are the mirror, the sub-stage condenser and the iris diaphragm where, as is most usually the case, the object is to be examined by transmitted light. For opaque objects which can only be usefully examined with low powers illumination comes from above the stage.

The Mirror is attached below the condenser. It has two surfaces, one concave and the other plane. The plane mirror is that employed for work with higher powers. Too small a mirror should not be used.

The Sub-stage Condenser.—This is placed between the mirror and the stage and collects the rays of light

received from the mirror into a cone of large aperture, which can be focussed on to the plane of the object.

It must be centred so that the optical axis corresponds with that of the objective, and must be movable so that it can be moved up or down in this axis. The movement is better performed by a rack and pinion, but in most of the portable microscopes has to be done by hand.

To the tube are fixed at each end the two systems of lenses used for the magnification of the object. The lower system of lenses, which is screwed on to the lower end of the tube, is the objective and forms a real image of the object, which is further magnified by the system of lenses at the upper end of the tube—the eye-piece.

To save time, annoyance and wear of screws a nose-piece is fitted to the lower end of the tube, to which can be screwed the three objectives in use instead of screwing them directly to the lower end of the tube.

These are the essentials of a microscope for the work here contemplated. It can be purchased complete for about £20 from several well-known makers. The price varies a little, but the reader is strongly advised to pay little attention to slight differences of price in the selection of an instrument that suits him. Much more expensive instruments can be purchased, but at about the above-mentioned price an instrument can be obtained suitable for the work contemplated. The portable microscopes with the same objectives are about £3 or £4 less.

No microscope should be bought without spending some time in careful examination and testing of the lenses and adjustments. The points to which special attention should be paid are: (1) As to the rigidity of the stand. This rigidity must be constant both with the tube vertical and inclined. (2) All the adjustments and screw movements must be tested to see that they work smoothly and evenly and that every movement of the milled head

results in effective movement of the screw and of the part of the instrument which it is intended to move.

With the mechanical stage it is further necessary to satisfy oneself that the movement imparted to the stage is all in one plane, otherwise as the object is moved it will also move out of focus.

This can be ascertained by examining an object, such as a uniform blood film, under various powers and determining how far the object remains in focus. When using a $\frac{1}{12}$-inch objective, even with the best stages, some focussing will be necessary, but it should be slight, and the object should be very little out of focus with considerable movements of the mechanical stage. A slide and film of uniform thickness must be used for this test, and the result of the examination should be confirmed by using a series of slides.

The nose-piece should centralise the objective correctly, otherwise an object that is in the centre of the field with a low power may not be in the field with a higher power. This is tested by centralising with the highest power some object that is visible with the lowest power, and seeing how near the centre of the field this object is when viewed with the other objectives. The order should also be reversed if the test appears to be satisfactory.

In testing the objectives the points to be most closely investigated are :—

(1) Definition. Unless the object is sharply and clearly defined the magnification is wasted.

(2) Flatness of field. Many lens give good and sharp definition at the centre of the field, whilst objects a little removed from the centre are blurred, and those at the periphery are out of focus. In using such lenses, if any other part of the field is brought into focus the objects in the centre of the field will be out of focus. With such a lens the field is not flat. It is perhaps too much to hope that the

periphery of the field will be in sharp focus when the centre is, but at any rate for blood work the greater part of the field must be flat, otherwise objects such as malaria parasites can easily be overlooked.

(3) Chromatic aberration must be entirely corrected and no particoloured fringe seen round the edge of the field.

(4) Magnification. As a test object a well-stained, evenly-spread blood film is as good an object as any other, and as the object is a familiar one the degree of magnification can be readily estimated. Both eye-pieces should be used in turn.

In the use of the microscope great attention must be paid to the illumination. The light in the Tropics is not good, as it so often has to be derived from blue sky. The mirror should be turned so as to receive the light from a white cloud when possible.

In using a low power the condenser should be low so as to be out of focus, or if the stand permits it, to swing out so as not to be between the mirror and the object.

With a $\frac{1}{6}$-inch objective it should be higher, and with the $\frac{1}{12}$-inch oil immersion objective close to the under-surface of the slide.

The brightest and most uniform light that can be obtained with the iris diaphragm open is the best. If we wish to reduce the light that should be done by closing the diaphragm, not by altering the position of the condenser or of the mirror.

Both the mirror and condenser should be kept clean.

It is well to have a spare mirror, as these silvered mirrors sometimes deteriorate rapidly in the Tropics.

In focussing with the microscope it is well to bring the objective nearer to the object than is necessary, and then, using the coarse adjustment, whilst looking down the microscope to withdraw the objective from the object till it is seen more or less distinctly through the

microscope. For exact focussing one uses the fine adjustment, but not till the object is nearly in focus. The range of the fine adjustment is small and if used over too extensive a range there is risk of straining it.

When working with the oil immersion it is well to place the oil on the object and screw down the tube till the objective touches the oil. In doing this the drop of oil should be viewed from the side and it will then be easy to see when the objective touches the oil. Then very slowly focus on to the object. Before using an oil immersion lens the field should be examined with a low power to make certain that there is something visible in the field. In a fresh blood film, for instance, if a part be selected in which there are no corpuscles there may be nothing to focus on to, and in such a case there is risk of screwing the objective down on to the object.

If black specks are visible in the field it is well to rotate the eye-piece ; if these rotate with the eye-piece they are particles of dirt in some part of the eye-piece. Dirt on the objective shows as a general haziness ; such haziness may also be due to a cloudy or dirty cover-glass or a badly prepared specimen.

All glass, and particularly the softer and more highly refractile glass of which lenses are made, is liable in a hot, moist climate to deteriorate and become cloudy or white, like very fine ground glass.

When lenses become affected in this way they require regrinding. Some lenses spoil more quickly than others, and in purchasing objectives it should be stated that they are required for work in the Tropics. Various less serious conditions are sometimes mistaken for this change in the glass. The cement may run so that it partly covers the inner aspect of the objective. In other cases water condenses between two lenses and causes a similar want of definition to that due to frosting of the glass. Either of these conditions may be detected by unscrewing the

lenses and examining the surface with a watchmaker's glass or hand-lens. These conditions, when discovered, are easily remedied. It is well only to use lenses that can be unscrewed, and from time to time to unscrew and clean the surface of the lenses carefully. They will keep longer if this is done, but must not be expected to last as long as they do in England. Lenses not in use are best kept in a perfectly dry stoppered bottle. There is no objection to having some dehydrating agent such as well-dried calcium chloride in a separate compartment in the same bottle.

A camera lucida or drawing camera is a great convenience, and so useful for measurements that some form of this instrument should be used. That of Leitz is a cheap and simple form, the use of which it is easy to learn.

For measurements a micrometer slide ruled to $\frac{1}{100}$ of a millimetre is a useful accessory; failing it any of the standard ruled scales, such as the counting chamber of a Zeiss' or Gowers' hæmocytometer, can be used as a substitute.

A micrometer scale to be placed in the eye-piece in focus with the front lens is useful for some measurements, but can be dispensed with if measurements are made with a camera lucida. A more useful form of eye-piece micrometer is ruled in squares. These can be used once they are standardised for blood counts and the ruled scales used for the counting chamber of a hæmocytometer, &c., dispensed with. For many purposes it is convenient to subdivide the field, and this can be more readily done with a micrometer eye-piece ruled in squares than in any other way. With such a micrometer eye-piece the ruling on the counting chamber of a hæmocytometer can be dispensed with.

These eye-piece scales are simply placed in the eye-piece and rest on the diaphragm between the two lenses.

The diaphragm will usually want moving a little for the scale to be sharply focussed, but this is easily done as the diaphragm can slide up and down inside the tube.

FIG. 4.

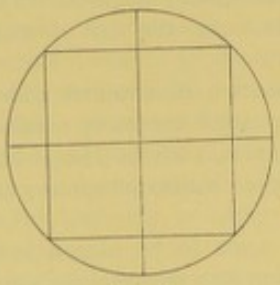


FIG. 5.

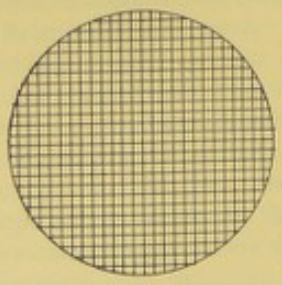


FIG. 6.

These eye-pieces require standardisation for the value of the squares or scale. The micro-millimetre scale is used as the object and for each objective the number

of micro-millimetres in a division of the scale noted. This can be done once for all and the records preserved.

There is no object in having the divisions of a scale or the squares of an accurately known size. As seen in the eye-piece they are magnified.

A warm stage is not so much needed in the Tropics as in England, but is a convenience. The simplest form is a copper plate perforated with a hole the size of a shilling. From the plate is a copper tongue extending in front for about six inches. The under-surface of the plate is covered with cloth and is placed on the stage so that the aperture corresponds to the central aperture in the stage.

The object is placed on the slide on the copper plate and examined, and by heating the tip of the tongue of copper projecting from the plate with a spirit lamp the heat will be conducted to the plate and the slide kept warmed. By heating the tongue nearer to the plate a higher temperature will be obtained, or by lowering the spirit lamp, or moving it further off, a lower temperature. With a little practice there is no difficulty in maintaining a fairly steady temperature which can be estimated by touch. More elaborate warm stages are to be procured in which the temperature is kept steady by the circulation of hot water.

A dissecting microscope is useful but not essential; it consists of a single compound lens which is fixed on a vertical carrier which can be raised or lowered by a rack and pinion. The stage is of glass and there are wooden movable hand-rests at each side.

For illumination there is a plane reflector, and as an alternative on the other side of the mirror a plaster of Paris disc.

For most of the purposes for which the dissecting microscope is used a watchmaker's glass does equally well, and for some purposes it is better.

A good large hand-lens on a handle is useful for observing the habits of mosquito larvæ.

Reagents, stains, slides and cover-glasses are required. The slides should not be of the best quality; the thin, white slides deteriorate more rapidly in the Tropics than the coarser glasses. No. 2 quality is to be preferred. They require thorough cleaning, and a stock cleaned and ready for use, requiring only to be wiped, should be kept in hand. They are best cleaned by placing in a saturated solution of carbonate of soda which is just brought to the boil. They are then well washed in running water, wiped with a soft linen rag, and placed and kept in spirit in a stoppered or well-corked, wide-mouthed bottle. Before use the slide must be taken out of the spirit and well rubbed with a soft, clean linen rag.

Cover-glasses are best sent out in oil or covered with oil, as even in the course of a voyage lasting two weeks they may become frosted. The whole mass of cover-glasses, say half an ounce, is placed in oil of cloves, and the cover-glasses are separated so that the oil penetrates between them. They are then taken out of the oil, wrapped in cotton wool and can be replaced in their boxes. Treated in this way they will keep for months even in the worst climates. They keep well also if sent out in spirit, but this is not recommended, as if the spirit evaporates completely the glasses deteriorate very quickly.

Cover-glasses treated with oil are not very easy to clean as the oil will have hardened and dried to a large extent. A good deal of the oil can be removed by placing the cover-glasses in 1 per cent. lysol solution or in xylol, and separating them or stirring them up. This saves the spirit which is necessary to more completely remove the oil. They must not be left more than a couple of hours in the lysol, and then should be placed and kept in spirit which will gradually remove nearly all the oil.

A small stock should be further prepared so as to be ready for immediate use. This may be done by first just bringing them up to the boiling point in a saturated solution of carbonate of soda, washing well, preferably in running water, and transferring them to strong sulphuric acid; in this they should be left over night, then again well washed in water and finally transferred to a wide-necked, well-stoppered bottle half filled with spirit. For use they should be taken out with forceps and well rubbed with a soft linen rag.

Such cover glasses should be free from both grease and grit and are then fit to use for making fresh fluid blood films or for other preparations. As alternatives to the treatment with carbonate of soda and sulphuric acid some prefer strong nitric acid and others bichromate of potash (2 parts), sulphuric acid (3 parts) and water (25 parts); others, again, sulphuric acid alone. In any of these solutions the cover-glass can be kept indefinitely and washed in water immediately before use.

Cover-glasses should be of the best quality, and for blood work the thinnest (No. 1) should be used. A smaller stock of thicker cover-glasses should be kept for the examination of fæces and making "squash" preparations. These thicker cover-glasses do not deteriorate so rapidly.

For bacteriological work some form of steam steriliser is necessary to sterilise vessels, media, &c. With this all requisite sterilisation for ordinary work can be done, but a hot air steriliser is an advantage for the quicker and easier sterilisation of vessels, Petri dishes and some instruments.

A steam steriliser (fig. 7) is simply a tall metal vessel covered with a lid with a vent for the escape of steam, and containing water at the bottom. As it is not well to immerse the objects to be sterilised in the water there is a perforated false bottom above the level of the

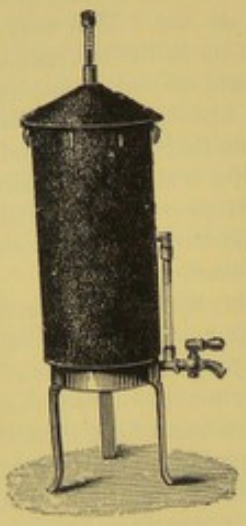


FIG. 7.

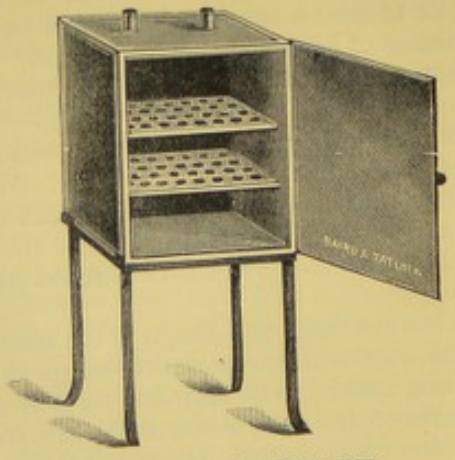


FIG. 8.—HOT AIR STERILISER.

water on which such objects rest. The whole vessel to prevent loss of heat is covered with some non-conducting material. There is no great difficulty in improvising such a steriliser, but those sold are more sightly and convenient.

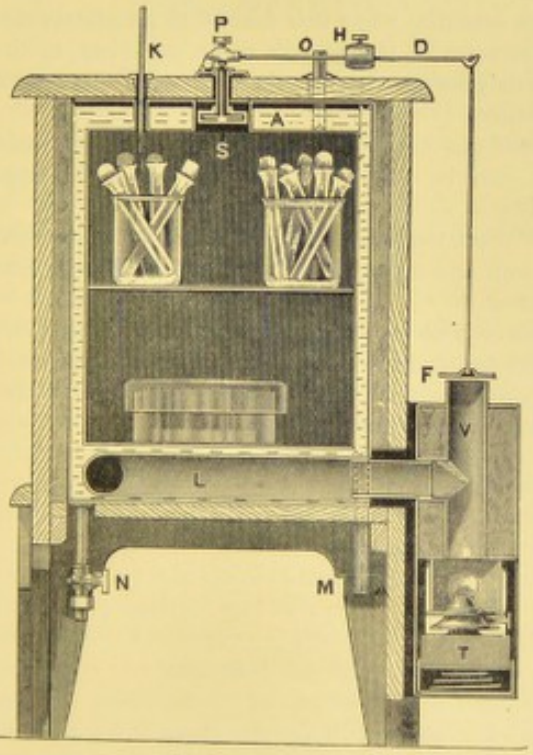


FIG. 9.—HEARSON'S INCUBATOR, WORKING WITH PETROLEUM LAMP.

They can be heated by a paraffin lamp, and the "Primus" is one of the best. At a pinch a wood or other fire may be used.

The hot air steriliser (fig. 8) is a metal case enclosed

in a second larger one, the two being separated by an air space. The double case can be dispensed with, but the heating is then less uniform. A temperature of about 160° C. is required.

Incubators are needed where it is important that growth should take place at a uniform temperature, and are essential when it is desired to accurately describe the character of the growth of an organism, or to compare one growth with another, or with the description of another.

Where gas cannot be obtained a form of incubator which can be used with kerosine must be employed (fig. 9).

At " room temperature " in the Tropics most organisms grow well and much useful work can be done without an incubator.

If there is no incubator a dark cupboard must be used, as light has a deleterious effect on most bacteria. This cupboard should be fixed in a dry place where the temperature is as uniform as possible.

CHAPTER II.

MATERIAL.—The methods for the preparation of blood films, fæces, mosquitoes, and bacteriological material are described under those headings. Other material is obtained at *post-mortem* examinations.

Post-mortem examinations in the Tropics present certain differences from these examinations in temperate climates.

Post-mortem changes are more rapid, so that it is essential that the examination should be made as soon as possible after death. This is not only on account of the rapidity of the putrefactive changes which occur, but also because many of the animal parasites die, and some, such as the sporozoa, disintegrate and cease to stain well even before putrefactive changes set in.

Certain special observations are worthy of attention :—

(1) The weights and relative weights of the organs, as these vary considerably from European standards, both when healthy, and as a result of disease. With the lungs, in recording the weight, it is essential to note also the time that has elapsed between death and the examination, as the weights of these organs increase a few hours after death, probably by aspiration of fluid. If the examination is made two or three hours after death the lungs will be barely half the weight taken as the standard in Europe, if the examination is made later the weights may be comparable.

(2) Variation of weight of the organs with age

differs in different races, and the curves obtained for the organs are in many cases different from those recorded in Europe. The brain weight in Europe attains its maximum between 45 and 50, whilst in the negro the maximum is reached between 20 and 25.

(3) The specific gravity of organs such as the liver, kidney and heart should be determined when there is evidence of fatty degeneration of these organs.

(4) *Abnormalities*. These are common, and some races, such as the Chinese, appear to have an unusual proportion of one, such as Meckel's diverticulum, whilst other races, as the Negro, more frequently have deeply fissured lungs. Disease also affects organs differently according to race, and of this the variation in the age incidence of splenic enlargement in Negro and other races living under the same conditions is a striking example.

(5) Abnormal appearances, such as congestion, ecchymosis, &c., are more common in the Tropics, and are observed under different conditions, as the examinations are made so much earlier. On the one hand, the appearances more closely resemble those in the living subject, and on the other hand, because putrefactive changes, particularly in the vicinity of the intestine, occur so early, patchy, irregular *post-mortem* staining is common and frequently mistaken for disease.

(6) Certain special putrefactive changes deserve close attention. As a result of putrefaction some of the organs, and particularly the spleen, often appear of a slaty colour which may be mistaken for malarial pigmentation. Section of the organ will show that the discoloration of early putrefaction only extends for a short distance from the surface into the substance of the organ. The substance of

the spleen in section sometimes appears very dark, but this colour can be distinguished from that of acute malaria by noticing that the dark colour changes to bright red after exposure to air. The only satisfactory test of malarial pigmentation of an organ is by examination of a portion of the tissue with the microscope. It is not necessary to cut sections, a small portion of the organ can be pulped between two slides and examined at once for pigment.

Another effect of *post-mortem* changes which may occur early is alteration in consistence and feel. To those who have not made such examination soon after death, healthy kidneys appear to be very hard, probably from *rigor mortis* of the cells.

Kidneys examined late, on the other hand, are flabby, but tough and not readily torn.

A diffluent spleen is often described, but is not met with in *post mortems* made early. The spleen, even in the most acute cases of malaria, though enlarged and black, is firm, and wedges of it can be cut with acute angles. These angles retain their sharpness even when exposed to a jet of water. Such a spleen is easily pulped, and if allowed to decompose speedily becomes "diffluent."

Many of the early putrefactive organisms form gas and consequently emphysematous changes; emphysema of the liver and other organs are common. In the intestines small emphysematous patches form in the submucosa and present a peculiar and rather deceptive appearance.

Gaseous distension of the whole intestine is very common, and the stretched walls appear unusually thin and are often described as atrophied.

Worms and intestinal parasites die as a rule within some six to twelve hours after the death of the host, and some, such as the ankylostome, lose their hold on the intestinal walls even earlier.

In the examination of intestines in the Tropics it is

not advisable to wash out the intestine before opening it. The intestine should be opened and examined for entozoa first and subsequently washed to see the condition of the mucosa. If the intestines are washed out first the entozoa will be carried away and may escape notice.

A large proportion of tropical diseases are those affecting the abdominal viscera.

To study the exact relationship of the parts it is often advisable to remove the thoracic and abdominal viscera *en masse*. The abdomen should be freely opened and room gained by subcutaneous division of the muscles attached to the pubes below, and sternum and ribs above.

The attachments of the diaphragm to the sternum and costal margins must next be divided with the knife close to the chest wall, and the parietal peritoneum stripped off the abdominal wall with the hand as far as possible. The trachea and vessels going to the neck are then to be freely divided by passing the hand and knife in front of the lungs and cutting transversely above the root of the lungs, whilst with the hand the thoracic viscera are grasped firmly at the root of the lungs and steady traction exercised. When the division is complete, the lungs and heart will be easily pulled downwards through the lower opening of the thorax. With the knife the posterior attachments of the diaphragm are divided from above, and steady traction, aided by a few touches with the knife, will strip the peritoneum off the remainder of the wall of the abdomen, and all the abdominal viscera with the aorta and kidneys will be completely separated except at their pelvic attachments. These can be divided, or better, the peritoneum stripped off the pelvis at each side, and the urethra and rectum divided as near the perineum as possible.

The mass of organs can be now examined from every aspect and the relations of the different parts readily observed.

By this method the root of the mesentery, the posterior mediastinum, and other parts which are usually overlooked can be displayed, and in these regions some parasites are found.

To prepare specimens of a whole organ or of a series of organs *in situ* they should be placed in formalin solution. Two per cent. solution is quite sufficient, but the amount of fluid must be in great excess and the intestines flushed out with the solution and injections made into the larger organs, such as the liver. The fluid should be changed in a few hours. A common mistake is to place the organ in a vessel that can only just hold it and add the solution of formalin so as to cover the organ. In estimating the strength of the formalin solution it must be remembered that water forms a large part of animal tissues and that consequently the formalin solution added is diluted by the amount of fluid in the tissue. Though formalin penetrates more rapidly than most preservative agents, decomposition will continue in the thicker parts of an organ unless it be freely incised, or have formalin injected into the vessels, or into the substance of the organ in several places with an exploring syringe.

The colours disappear or change in the formalin solution, but can be largely restored by placing the organ in strong spirit for twenty-four hours.

When the colour is restored the organ can be placed and kept for exhibition in a solution of glycerine and acetate of potash or other substances, and little further change in colour will take place. A good formula for this solution is glycerine 20 parts, potassium acetate 15 parts, and water 100 parts.

Any of the large worms, tape-worms, &c., should be placed in glycerine at once, whilst the smaller ones, such as filaria and ankylostomes, are best placed in 1 per cent. formalin, and are afterwards changed to a 2 per cent. solution.

MICROSCOPIC EXAMINATION.—If parts of an organ or tissues are to be preserved for microscopical examination they can be examined fresh, or preserved and hardened.

Much information can be gained from the examination of the fresh specimen, either by making smears of the fluids that exude from the cut surface, squashing small portions of the tissue between the slide and a cover-glass, or by freezing and cutting sections. In the last case they should be placed in a strong solution of gum arabic.

More often the specimens require hardening, and for most purposes alcohol is the best hardening reagent for tropical work, as parasites stain well after hardening in alcohol.

Specimens for microscopical examination must be hardened uniformly, and it is essential, therefore, that the pieces of tissue must be small and that the fluid can penetrate on all sides.

If a piece of tissue is simply put into a bottle and spirit poured on it, the blood coagulates at the edges of tissue where in contact with the glass and the fluid does not penetrate between the glass and tissue. This is avoided by placing some cotton-wool at the bottom of the bottle, or small pieces of crumpled paper.

For fixation of tissues alcohol is the most useful reagent in tropical work, as the parasites of malaria stain better after fixation in alcohol than in any other reagent. It has the disadvantage of causing great shrinking of the tissue and parasites.

To fix in alcohol, the tissue must be in small cubes not more than half an inch in their greatest length, and placed in at least ten times their volume of alcohol in a closed glass vessel, with some cotton-wool at the bottom, and left for six hours.

In certain cases, especially where it is desirable that the blood should be retained in the vessels, a larger

piece of tissue can be taken, and after this partial fixation subdivided into pieces of the right size. This is particularly to be recommended when the object is the examination of the tissue for malaria parasites or filaria *in situ*. At the end of six hours the alcohol should be changed, and again changed in twelve hours. By this time in a warm climate the specimen will be sufficiently fixed, and longer immersion in absolute alcohol will render the specimen too brittle. In colder weather, average temperature under 70° F., it can be left some hours longer in the alcohol.

The specimens when fixed can be kept till required in methylated spirits, which in the Tropics are usually weaker than in England. If greater accuracy is required the specimens can be kept in 60 per cent. absolute alcohol. This will keep the specimens, and stronger alcohol at tropical temperatures soon overhardens them.

For more rapid fixation of tissues in which examination for malaria parasites is not required, alcohol and formalin give excellent results. This solution is made by the addition of formalin in the proportion of 2 to 10 per cent. to the absolute alcohol. It penetrates rapidly and causes less shrinking than alcohol alone, but the tissues should not be left in this solution for more than twelve hours or they will be overhardened. They are then fit for further processes or can be kept in spirit.

Müller's Fluid.—Pot. bichromate 2·5 parts, sodium sulphate 1 part, and water to 100 parts is very extensively used and gives good results, but is slow in its action. The fragments of the tissue are placed in abundance of the fluid, which should be changed in a few hours, and again daily for a week, after that once a week will be sufficient. Some tissues will be sufficiently fixed in two or three weeks, but others, as the parts of the central nervous system, may, even in a warm tropical climate, require many weeks. When fixation is complete the

specimens should be washed for twenty-four hours in abundance of water, which is frequently changed, preferably in running water, and then kept in methylated spirit.

ORTH'S FLUID.—Müller-formol is made by adding 10 per cent. of formalin to Müller's fluid. This must be added immediately before use. It is a rapid fixative, and at blood heat only three or four hours are required for thin pieces of tissue. Two days are usually sufficient at room temperature.

Parasites do not stain well in tissues which have been fixed in bichromate solutions.

For the examination of skin, which is readily overhardened, Zenker's fluid gives good results. This is composed of 5 parts of corrosive sublimate, 2·5 parts of potassium bichromate, 1 part of sodium sulphate, and 100 parts of water. The slices of tissue to be examined must be very thin, not more than a tenth of an inch in thickness. The time required for fixation is twelve to twenty-four hours, according to the thickness of the specimen and the temperature.

After the tissues are fixed they must be thoroughly washed in water, which is frequently changed for at least twelve hours, and should then be placed in spirit to which a little tincture of iodine has been added, as this removes any mercury deposited in the tissues. If the colour of iodine disappears from the fluid more iodine is to be added until the colour no longer disappears. Or the specimens can be kept in spirit and the cleaning with iodine done after the sections are cut. The specimen can then be kept in spirit till required for use.

Two other useful fixatives are, Flemming's solution :—

Osmic acid 2 per cent. aqueous solution	4 parts ;
Glacial acetic acid	1 part ;

Chromic acid 1 per cent. aqueous solution .. .. 15 parts;

and Hermann's fluid :—

Osmic acid 2 per cent. aqueous solution .. .. 1 part;

in which a 1 per cent. solution of platinum chloride is substituted for the 1 per cent. solution of chromic acid in Flemming's solution.

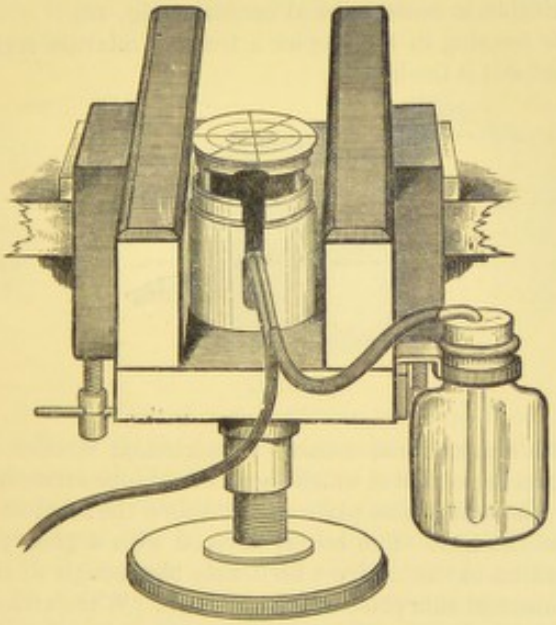


Fig. 10.—Microtome, Cathcart's, with Spray Bellows.

These solutions must be freshly made up before use, and as the penetrating power of the fixative is low the specimens must be very thin, not more than $\frac{1}{12}$ of an inch in thickness.

Fixation takes from one to two days, and the specimens

require to be thoroughly washed, preferably in running water, for one day, and then placed in 80 per cent. alcohol.

To Cut Sections.—Sections can be cut with a sharp razor. To cut sections by hand requires much practice, and only very small pieces of tissue can be cut satisfactorily in this manner.

Cathcart's microtome is the simplest efficient instrument, but its use is limited, as freezing with ether is not practicable in most tropical countries (fig. 10).

For freezing in the tropics a freezing mixture such as ice and salt is the best.

Fig. 11.

Swift's microtome consists of a circular wooden box (A) from the centre of which rises a metal tube surmounted by a horizontal zinc plate raised above the level of the top of the box. The box is covered with a glass plate perforated in the centre with a hole big enough to allow the tube and zinc plate to pass through. When arranged for use the box is filled with a mixture of well-crushed ice and salt. The lid is placed on and the zinc plate projects above its level. The substance to be frozen is placed on the zinc plate and well covered with a strong solution of gum. If the air temperature is not too high the specimen freezes with the surrounding gum (fig. 11).

In many tropical countries this does not suffice unless

the specimen is also surrounded by a cold atmosphere. This is produced by placing a second metal box (B) on the top of the glass plate. This metal box has a central tube rising from the bottom and open below, and this tube must be wide enough to allow the zinc plate and specimen to be inserted in it. If the metal box is also filled with the freezing mixture the air in the central tube will be cold and the specimen surrounded by this cold air freezes readily (fig. 12).

When frozen the upper metal box can be removed and sections cut.

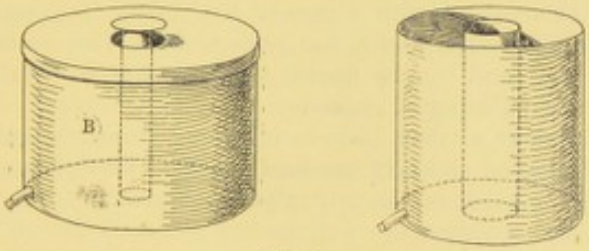


FIG. 12.

In this instrument the specimen remains fixed, and the thickness of the section is regulated by alterations in the level of the razor. This is arranged by having the razor blade fixed on a tripod; the length of the legs of this tripod can be regulated by turning the milled heads of the screws. The feet of the tripod are tipped with bone so as to slide evenly over the glass. For use the blade of the razor must be wetted with water and the tripod carrying it is so arranged that one leg is anterior. The two posterior screws are turned till the edge of the razor is horizontal or parallel with the surface of the glass. Any alteration in the screw of the anterior leg will then raise or lower the edge of the blade.

The sections are cut by gliding the tripod over the

plate till the edge of the razor touches the specimen and cuts through it. A slightly oblique motion is the best, and the tripod must be pressed firmly on the glass plate and the movements must be rapid, more like a thrust. The knack of making the correct movement is soon acquired.

When a section is cut the tripod is drawn back, slightly tilting it to avoid touching the specimen, the anterior screw turned to an amount regulated by the thickness of the desired section and again thrust forward. This process should be repeated until there are several sections on the upper surface of the blade of the razor, and these can be removed with a camels' hair brush to a vessel containing water which has been boiled. The sections will float and can be floated on to a slide and either examined directly or stained.

For these sections picro-carmine and Bismarck brown may be used and give excellent results. The specimens may be mounted in any glycerine medium such as Farrant's solution.

Sections of fresh, unfixed tissues can be cut and examined unstained, or, to show structure better, they can be stained. They should be soaked in gum before freezing.

Well-fixed specimens can also be frozen and cut. It is necessary before freezing to thoroughly remove the last traces of alcohol by washing in water, and to soak in an aqueous solution of gum arabic for some hours. Where ice cannot be obtained further hardening and imbedding is necessary.

There are many processes, but two only are in general use. In these processes the piece of tissue is imbedded in some easily cut solid substance, which must also permeate through the whole tissue. Paraffin wax and celloidin are the two substances most generally used. With paraffin thinner sections can be cut, but good sec-

tions of only small pieces of tissue are obtainable, and for some purposes paraffin is not well adapted.

To Imbed.—The general principle is to pass the specimen through alcohol till it is thoroughly dehydrated, then to place it in a fluid in which paraffin is soluble, which will dissolve out the alcohol, and then to replace this fluid by first a weak solution of paraffin, then a strong solution of paraffin, and finally melted paraffin wax. Excess of paraffin is poured round the tissue and it is allowed to cool, when the paraffin solidifies not only is the piece of tissue enclosed in a solid block of paraffin wax but the tissues will be permeated with the wax.

There are many modifications, some of which are rendered necessary for special tissues.

For general work with specimens taken from strong spirit :—

(1) Place the specimen in absolute alcohol for twenty-four hours. If the specimen has been removed from weaker spirit or from water, before placing in the absolute alcohol it should be placed in methylated spirit for forty-eight hours.

(2) Remove from spirit, drain off excess of spirit for a few minutes and place in aniline oil. One day.

(3) Place in xylol. One day.

(4) Place in paraffin and xylol, equal parts. One day.

(5) Place in melted paraffin wax for one day. The paraffin wax can be kept melted in a drying oven (fig. 13) at the required temperature, or a paraffin embedding bath can be used for this purpose (fig. 14). As a considerable amount of spirit is required for the spirit lamp to maintain the required temperature, it is well to imbed as many specimens as possible at the same time. The imbedded specimens keep well.

(6) Imbed and cool quickly.

The imbedding may be done by filling small paper boxes with melted paraffin and placing the pieces of tissue in this melted paraffin. The box is then placed in a dish of cold water on which it floats and is rapidly cooled so that the paraffin sets without crystallising.

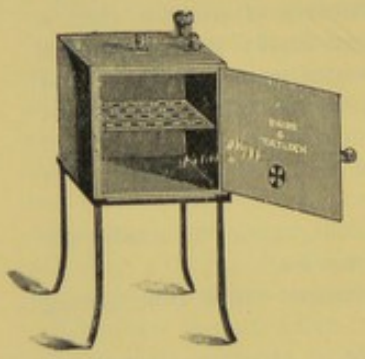


Fig. 13.

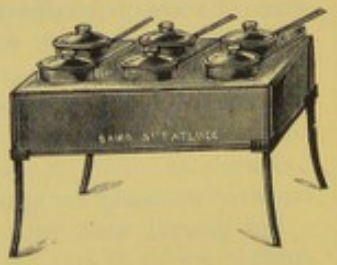


Fig. 14.

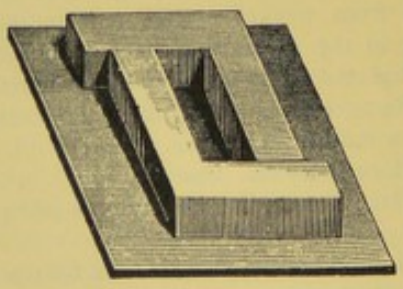


Fig. 15.

Or L-shaped pieces of metal are placed in contact on a smooth slab, as in the diagram (fig. 15), and the space between filled with the melted paraffin and the specimens placed in as before.

Modifications.—The paraffin used in England melts at too low a temperature for satisfactory work in the Tropics.

It is well therefore to keep two varieties of paraffin, one melting at 48° C. and the other at 60° C., and to use a mixture of them. Such a mixture with a melting point about 54° C. is usually sufficient, but in the warmest weather either a larger admixture of the paraffin at the higher melting point will be required, or the pure paraffin melting at 60° C.

Celloidin is indispensable when it is desired to keep any loose bodies *in situ* in a tissue, as there is no necessity to remove the celloidin before mounting in Canada balsam.

It is also useful for general work, and then the celloidin can be removed if it is thought desirable.

To imbed in celloidin the general principle is the same as that for paraffin, but the agents employed and the methods differ.

(1) The specimen is kept in absolute alcohol, after being in weaker spirit, for twenty-four hours.

(2) It is then soaked in a mixture of equal parts of ether and absolute alcohol for twenty-four hours.

(3) Place in a weak solution of celloidin (3 per cent.) in alcohol and ether for twenty-four hours or more; two days is usually ample.

(4) It is then to be transferred to a thicker celloidin solution, 6 per cent. celloidin dissolved in alcohol and ether, and kept in this for at least one day, and better for several days.

(5) The specimen is then placed on a small block of wood on which a few drops of the thick celloidin have been placed. Leave exposed to the air for a few minutes and pour a little thick celloidin solution over the specimen. Expose to air for a few minutes and place in 60 per cent. alcohol, which will harden the celloidin. In cutting celloidin specimens the knife must be oblique and must be moistened with spirit.

Modification (Stepanow's method). Oil of cloves can be used instead of alcohol and ether, and will remove the alcohol completely in about six hours. In this case the celloidin solution contains oil of cloves.

Formula :—

Celloidin (dry)	1·5 grammes.
Oil of cloves	5 cc.
Ether	20 cc.
Absolute alcohol	1 cc.

This penetrates the tissue in six hours or more.

The block of tissue with enough of the solution to cover it is poured into a filter of fine paper and kept in a warm place to allow the solution to thicken. Mount in the usual way and harden in 60 per cent. alcohol.

To Cut and Stain Sections of Mosquitoes.—According to Dr. Low, the best method is to kill the mosquitoes by dropping them into 60 per cent. alcohol alive, so that some spirit may be drawn into the interior. Keep them five days in this spirit. Remove the wings and legs from the mosquito and place the trunk in 95 per cent. alcohol for twenty-four hours, then in absolute alcohol for twenty-four hours, then in alcohol and ether equal parts twenty-four hours. After this thin celloidin one day. Thick celloidin one day. Mount on blocks, hardening the celloidin on those in 60 per cent. spirit; then cut serial sections, keeping the sections in 60 per cent. spirit. For staining float out in water. Stain in watch glasses with hæmalum or hæmatoxylin for five to ten minutes so as to overstain decidedly. Decolourise with 1 per cent. hydrochloric acid in 70 per cent. alcohol till when replaced in water only a faint violet colour is retained by the mosquito. Replace in 60 per cent. spirit, then in 95 per cent., and from that to carbol-xylol 25 per cent. till the section appears perfectly clear and transparent. Transfer to slide, press firmly with clean filter paper, and mount in xylol balsam.

Imbedded sections can be cut with the freezing microtome, but must not be frozen. The carrier is heated and the paraffin block pressed against it. The paraffin will be melted and will then adhere to the zinc plate; but better sections can be obtained with other microtomes.

Of the simpler and cheaper forms of microtomes the Cambridge Rocker (fig. 16) is the most convenient. A form of this instrument should be selected in which the

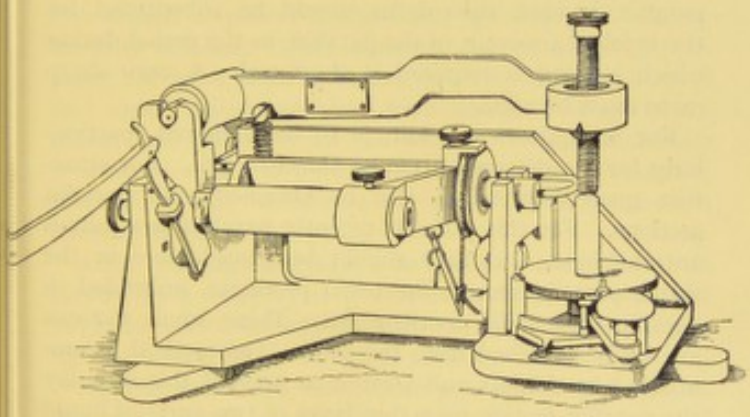


FIG. 16.—CAMBRIDGE ROCKING MICROTOME, NEW PATTERN FOR CUTTING FLAT SECTIONS, WITH LARGE ARTICULATING APPARATUS AND ONE RAZOR.

razor can be placed obliquely, as otherwise specimens imbedded in celloidin cannot be cut satisfactorily.

Full directions are sent for the use of this instrument with the microtome, but the chief points to observe are :—

(1) That the razor must be rigidly clamped.

(2) That the paraffin must be firmly fixed on the metal carrier. This is done by heating the carrier and applying the paraffin block firmly to it and keeping it in position till the carrier is cold.

(3) Graduate the thickness of section in accord-

ance with the nature of the tissue, its brittleness, and object of the section. If the specimen is too hard or brittle it is useless to expect thin sections.

Sections showing large parasites such as filaria in tissues like the lung should not be too thin. For the details of nerve structure, and for sections showing bacilli, or the parasites of malaria, the thinnest possible sections are the best.

For skin sections celloidin is to be preferred. If paraffin is used chloroform should be substituted for the xylol as a solvent of the paraffin, as the period during which heating is required is shortened. A very sharp razor must be used.

For mosquitoes celloidin is to be preferred, particularly for the demonstration of filaria *in situ*. For structure good results can also be obtained with paraffin sections. For this purpose recently hatched mosquitoes are the best, and they should be placed alive in the spirit, passed through the usual processes, imbedded in paraffin and serial sections cut. These small sections are easily detached from the slide. The method recommended by Annett and Dutton to prevent this is to lay the paraffin section on a thin layer of two parts of liquid glucose and one part of a thick syrup of pure dextrin spread on a slide and kept in the hot incubator till the glucose mixture has dried hard. The paraffin is then removed by xylol and alcohol and a solution of photoxylin is poured over the slide so as to form a film over the sections. This is allowed to set till the edges of the photoxylin film crinkle. On placing the slide in water the film comes away with the sections which can then be stained in the usual way. Carbol-xylol must be used for clearing.

Fixation of Sections in Paraffin on Slide.—For ordinary work the sections, when cut, are placed on the surface of some warm water about 44° C., or rather more if paraffin of a higher melting point is used, and floated

on to a slide. The water is allowed to drain off and the slide is then placed in the hot incubator for twelve to fifteen hours. The section will then be fixed to the slide.

Occasionally it will be found that the sections after removal of the paraffin fall off. In such a case the other sections may be very gently warmed over a flame till the paraffin *begins* to appear more translucent.

To remove paraffin from the sections so that aqueous and other stains can be used, the slide carrying the section should be placed in xylol and agitated in it for two or three minutes. This dissolves the paraffin. To remove the xylol place in strong spirit or absolute alcohol and again agitate so that fresh surfaces of spirit are brought in contact with the section. As a precaution it is well to rinse in fresh spirit. The slide can then be placed in water to remove the spirit and stained as is considered advisable.

After staining, dehydrate in alcohol, clear in oil of cloves, wash with xylol if aniline stains are used, and mount in xylol Canada balsam. If the alcohol used is strong enough the oil of cloves need not be used.

Most of the stains, and where possible other reagents, should be imported in solid or concentrated form and the bulk should be kept in that condition. There are several reasons for this. A considerable sum is saved in packing and carriage. Most stains keep better in the solid form, and if any bottle is broken the damage to other articles is less.

Some substances, such as methylated spirit and absolute alcohol, must be imported in bulk. As a substitute for methylated spirit, where sugar factories and distilleries occur, the crude spirit, "high wines" or "white spirit," can often be used. This spirit can be conveniently concentrated by abstraction of water with anhydrous copper sulphate. As this proceeding is often required for methylated spirits and can be economically

used to restore to a good strength alcohol that has been used for dehydration it is an important one. Crystals of copper sulphate are heated on a plate or in a dish, preferably copper, till they are nearly red hot. The water of crystallisation is thus driven off and the copper sulphate reduced to a white powder with a mere faint tinge of blue. This copper sulphate absorbs with great avidity the requisite amount of water of crystallisation, abstracting it from its mixture with the alcohol.

All, therefore, that is required is to add this anhydrous copper sulphate, prepared by heating the blue copper sulphate, to the spirit, shake it up well and allow it to stand. The water will be removed and the alcohol rendered more absolute. If the copper sulphate rapidly becomes blue more anhydrous sulphate should be added till the anhydrous copper sulphate remains nearly white in the spirit. The spirit is then decanted off and the copper sulphate can be used again after heating to expel the water.

A good pair of scales turning with ·05 of a gramme will be needed, a measure glass measuring up to one-half litre, as well as smaller measures up to 10 cc. English measures and weights can of course be used, but are less convenient.

Several ordinary iron glazed jugs and other vessels are very useful as they do not rust, are not easily broken, and will stand heat. Photographic trays of the same material can be obtained and are useful for washing specimens and floating out sections. Other photographic trays, half-plate size, are useful for breeding mosquito larvæ. For breeding and keeping mosquitoes suitable cages must be provided.

A full list of the apparatus that is of most use is appended at the end of the book.

CHAPTER III.

Blood.

Examination of the blood is of such importance in tropical work that in all cases of difficulty and doubt resource to this method of diagnosis is essential. A thorough knowledge of normal blood is a necessary preliminary. The abnormal forms of cells met with in various diseases must be readily recognised. Last, but not least, the various methods used for the finding and recognition of parasites must be known. Many methods of examining blood have been employed and most of them are good. Fallacies and mistakes have occurred with all, and the sources and causes of these errors and the recognition of them have to be studied. These are dealt with under each method described.

Blood is composed of a nearly colourless fluid, the plasma, in which are floating cellular elements, the red and white blood corpuscles and blood-plates. The more solid elements, the blood corpuscles, will be considered first. They vary in number, in their relative proportions and in their characters.

Other cells, not normally present in the blood, are found under certain conditions in that fluid. Most of these cells are normally present in the tissues of the body in health.

Parasites occur in the red corpuscles and in the plasma, and are sometimes found, in a more or less disorganised condition, in white corpuscles or phagocytes, which have

devoured them. None have been observed in the blood-plates.

The two main methods employed for the examination of blood are :—

(1) In the fresh and fluid condition.

(2) As films which are allowed to dry, and fixed and stained in various ways.

These two methods are of general application. For special purposes, so as to reveal abnormal bodies scantily present in blood, thick films can be employed and the hæmoglobin removed. In this way a quantity of blood that would not be sufficiently transparent if treated by the ordinary methods can be examined rapidly.

Examination of Fresh Blood.—This method is the only one by which vital changes can be observed. Of the normal blood elements, the amœboid and phagocytic properties of the leucocytes can thus be observed. Living organisms abnormally present, such as filariæ, trypanosomes, and the hæmosporidia or parasites of malaria, can be watched, and such developmental and degenerative changes as occur in shed blood observed.

No description or observation of new parasites is complete without an examination of these parasites in the living conditions.

It is noteworthy that most of the important mistakes made even by experienced observers in the description of bodies met with in blood have been due to neglect of the examination of fresh fluid blood.

The essential point in the preparation of fresh fluid blood films is that a great part of the film should be so thin that the blood corpuscles are lying flat and separate from each other. The simplest method of making such a film is to take a small drop of blood on the centre of a cover-glass and drop it on the slide (fig. 17). In a well-made film by this method three zones are apparent. The edge of the film is thick and irregular. Here the

corpuscles are in rolls or masses and it is too thick for the examination of the individual red corpuscles (fig. 17*a*).

Internally to this is an area with a slightly opaque or ground-glass appearance. Here the red corpuscles will be found lying flat and not to any great extent overlapping each other. This is the part of the film best suited for examination of the red corpuscles and the parasites contained in them (fig. 17*c*).

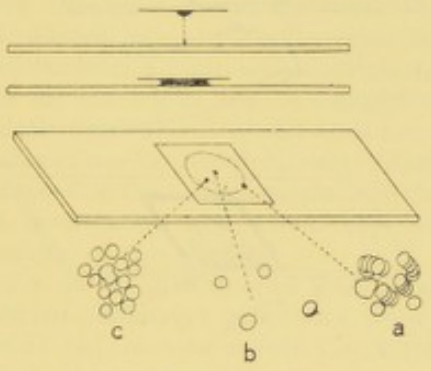


FIG. 17.

The centre of the film is clear and transparent, and here few corpuscles are found as this part is too thin and composed entirely of serum (fig. 17*b*).

To get good films by this method the slides and cover-glasses must —

(1) Be free from grease, as otherwise the blood will not run (*vide* cleaning slides and cover-glasses).

(2) They must be free from grit ; this is best done by rubbing well, immediately before use, with a soft linen rag.

(3) The drop of blood must be so small that, when spread out, it does not extend to the edges of the cover-glass. If the blood is too abundant it floats

up the cover-glass, and sufficient space is left between the slide and cover-glass to allow of the formation of rouleaux.

Another method is that of Braddon. Here the cover-glass, freed from grease and grit, is placed on a slide

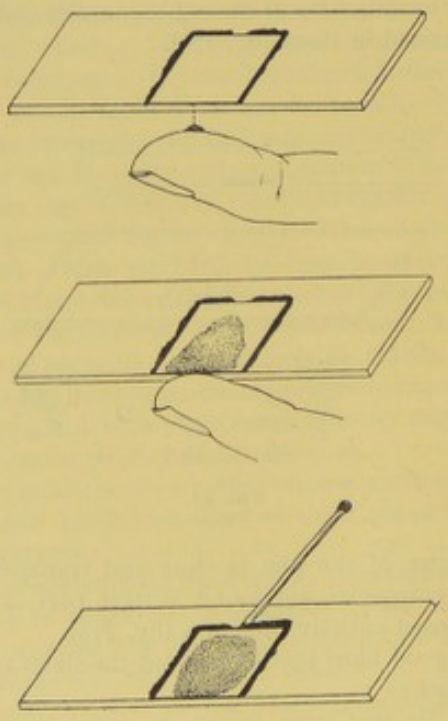


Fig. 18.

similarly cleaned. The cover-glass is so placed that its edge corresponds with one edge of the slide. Pressure is exercised on the centre of the cover-glass, or it can be fixed with Cornet forceps. Vaseline is then applied to the slide at the edge of the cover-glass, leaving the edge

free where it is applied to the edge of the slide and a small space at the edge opposite to this (fig. 18).

These slides can be prepared in the house or laboratory and are then ready for use. If the edge of the slide be applied to a drop of blood, the blood will run up by capillary attraction and spread itself in the space between the cover-glass and slide in a film thin enough for examination.

A third method is to make the film between two cover-glasses, the lower one being much the longer. The blood spreads more readily between two cover-glasses than between a slide and cover-glass, and the free edge of the lower glass can be clamped on to the slide (Horder's method). Beautiful films are obtained, and cover-glasses can be carried in larger numbers than slides on account of the smaller weight, but the greater fragility of cover-glasses is a serious objection to the general adoption of this method.

Any of the above methods give good results. The first has decided advantages in that the blood elements are all present and, to some extent, distributed evenly throughout the best part of the film. The second is very convenient for class work, as there is no delay at the bedside, and a large number of preparations can be made quickly. It is useful with nervous patients as no preparation is necessary at the bedside. The more adhesive elements of the blood, the blood-plates and leucocytes, are crowded together near the edge where the blood has entered. In the thinner part of the field, which is farther from the edge, these elements have been "filtered out" and few solid elements but the red corpuscles are left.

In the freshly-drawn blood the elements normally present are :—

(1) Red corpuscles, erythrocytes or xanthocytes.
(2) White corpuscles or leucocytes.

(3) Blood-plates or platelets, Hayem's hæmatoblasts.

(4) Plasma.

In the red corpuscles the points to note are the colour and the variations in colour. The size and variations in size and the shape. In many of the corpuscles, particularly if pressure has been applied to the cover-glass, clear, transparent spaces, vacuoles, which may be either circular, oval, or even slit-shaped, will be found. These must be recognised as what they are and not confounded with unpigmented parasites nor with the natural deficiency in colour seen towards the centre of the corpuscles. From both of these the vacuoles can be distinguished by their sharpness of the outline. An oscillatory or vibratile motion of the hæmoglobin edge of the vacuole is highly characteristic, but must not be mistaken for amœboid movement.

Crenation.—If the blood corpuscles be watched for some time they will be seen to become distorted and projections are thrown out, either as a few blunt processes or as sharper projections. This change is known as crenation, and the projection may be feebly motile and portions may break off and be discharged into the plasma. These crenations are readily recognised when they occur at the edge of the corpuscles. When they occur on the flat surface they produce an irregularity in the colouring and, by causing refraction, produce an appearance of dark spots surrounded by a lighter ring, or a light spot surrounded by a dark ring, according to the focussing.

Some of the red corpuscles, particularly if pressure has been used in making the film, are bent on themselves or "buckled." Such corpuscles may assume very varied shapes and, as the hæmoglobin is readily expressed from any part of the corpuscle, compressed irregularities in colour are usual.

Leucocytes are distinguished by their size and the

absence of colour. As seen in the fresh blood they are usually granular, the granules being best seen on closing the iris diaphragm of the microscope. Variation in the granules will be noted, and the coarse, highly refracting granules of the eosinophile leucocytes are quite characteristic. These granules are often mistaken for pigment by beginners. Letting in more light, which brings out pigment granules more strongly and shows these normal granules to be translucent, will remove this difficulty. The characters of the nuclei and of the granules are best studied in stained specimens. The amœboid movements and phagocytic properties are best seen in these fresh living fluid films.

THE BLOOD PLATELETS are the most difficult objects to see, as they are colourless, non-granular and differ little in refractive index from the plasma.

The size and arrangement in groups, points that vary in different specimens of blood, should be noted. The irregular serrated margins they acquire in a short time, from the formation of filaments of fibrin, are characteristic of these bodies. These elements are more readily seen in stained or over-stained specimens.

Many methods of staining blood, whilst still in a fluid condition, by admixture with stains have been employed.

The usual practice is to place a drop of sufficiently dilute stain on the slide, then take a minute drop of blood on the cover-glass and drop this on the drop of stain, so that the blood and stain spread out together. A certain admixture takes place at the edge of the drop of blood and in a little time the stain diffuses further into the blood.

Various solutions of stain have been used. Braddon's is perhaps as good as any.* In this, as well as in other

* Braddon's solution is composed of 1 per cent. pot. citrate, ½—2 per cent. methylene blue. Water to 100 parts.

aqueous stains, the water causes a liberation of the hæmoglobin, and the dissolved hæmoglobin precipitates the stain, or *débris* is stained by the stain.

If this process takes place in the serum little confusion is caused, but if, as frequently happens, it takes place as the stain penetrates the red corpuscles it causes the formation of a complicated stained arrangement in the interior of the red corpuscles, which has been mistaken for parasitic growth. To avoid this error, a strong salt solution is used by some so that the red corpuscles are not destroyed. Others, for the same reason, use ascitic fluid.

Malarial parasites are well stained by this method, and it has the advantage of requiring no fixation and consequently is rapid.

Dried Films.—These can be made in many ways, most of which after little practice give excellent results. In all methods it is important that the skin should not be touched by the slide, cover-glass or paper, but only the top of the drop of blood. Neglect of this precaution will result in the admixture of epithelial scales with the film.

(1) In this method a drop of blood is taken on the surface of a slide near one end. The edge of another slide is brought into contact with this drop which then spreads out so as to fill the angle between the two slides, the whole extent of the parts in contact. On pushing the upper slide towards the other end of the lower slide a film of blood will be left behind. The thickness of this film is easily regulated as, if the angle between the two slides is acute, the film left behind will be very thin. If the angle be near a right angle a thick film will be left. An angle of about 45° gives the desired thickness, but it is well to slightly vary the thickness of the film by alternately slightly increasing and diminishing the angle made between the two slides as the upper one is pushed

along, so that different parts of the film will be suitable for examination for different purposes.

A slight modification of the method is to take up the drop of blood on the edge of the upper slide and bring this drop of blood and the edge of this slide into contact with the upper surface of the lower slide and proceed as above (fig. 19).

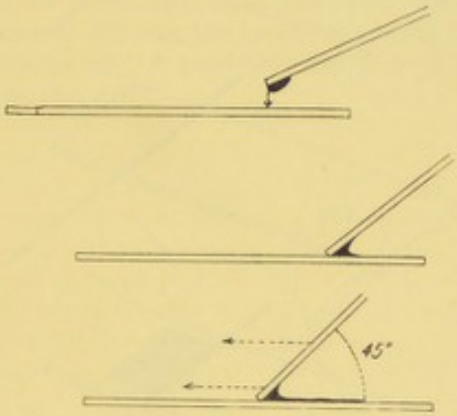


FIG. 19.

(2) A drop of blood is taken on a slide rather nearer one end than the other, and the larger the drop the farther from the middle. Another slide, a glass rod, or, perhaps best, the shaft of a needle is then applied to this drop so that the blood spreads along the whole of the line of contact. The upper slide, glass rod, or needle is then drawn across the lower slide and an excellent film will be left (fig. 20).

(3) Cigarette paper, or gutta-percha cut in the form of a narrow slip, is used in this method. The lower surface of the slip is brought into contact with the drop of blood on the finger or ear. This drop adheres to the slip on

removal. The edge of the slip is placed on the slide and the blood then spreads out between the tissue paper or gutta-percha tissue, and on pulling the free end of the

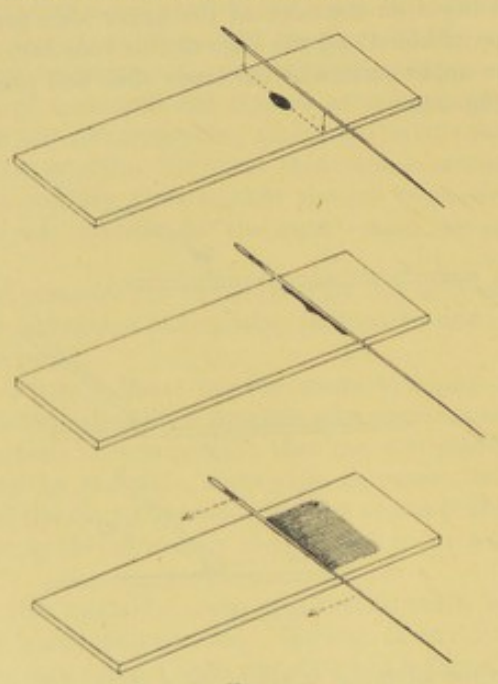


FIG. 20.

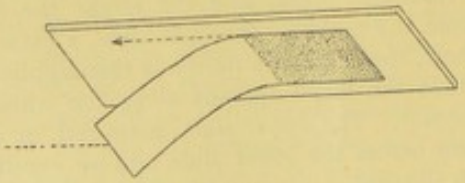


FIG. 21.

slip a good but usually scratchy film will be left (fig. 21).

These three methods can be used for cover-glass pre-

parations, particularly if long cover-glasses be used, but are best for slides.

(4) The last method advocated is not satisfactory with slides but is useful for cover-glass preparations.

A small drop of blood is taken on one cover-glass and this cover-glass is then applied to a second so that the blood spreads out between them. The cover-glasses are arranged diagonally so that the corners of each cover-glass can be taken hold of. The upper cover-glass is then drawn or slid over the lower, care being taken that it is not *lifted off*. A good film should be left on each cover-glass (fig. 22).

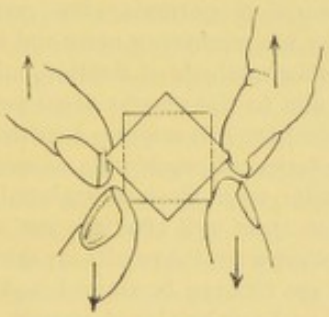


FIG. 22.

In case of emergency any piece of flat glass, broken window-pane, &c., can be used and good films obtained, but the best films are those in which the slides are of good quality, even in thickness and free from scratches, dirt, grease or irregularities.

The films, however made, should be dried rapidly but not heated, by waving them to and fro in the air. Otherwise crenation and distortion of the corpuscles will take place.

FIXATION.—If such films were placed in water or

aqueous solutions of stains the hæmoglobin would be dissolved out and the corpuscles more or less destroyed. It is therefore necessary to fix the films.

Films can be fixed by heat, but a temperature above a certain point vacuolates and distorts the red corpuscles.

As a general rule in blood work, fixation by heat should be avoided, though for one method of staining—the Ehrlich-Biondi—fixation by heat is necessary. Good results are more difficult to obtain in the Tropics by this method than in England, and, as the same information can be obtained by easier methods, it is not recommended for tropical work.

Fixation by absolute alcohol, or by absolute alcohol and ether in equal proportions, gives good and reliable results. Fix for ten minutes or more and then dry in air.

There are other methods of fixation, and of these exposure of the film to the vapour of 40 per cent. formaldehyde (formalin) for two minutes is perhaps the best.

Saturated solution of perchloride of mercury does not give good results with films of malarial blood as the parasites do not stain well after the use of this reagent, but for other blood work the results are fairly satisfactory.

When fixed the film can be stained, and the number of stains that have been employed is very large. Of the methods most generally applicable, the following have the special advantages and drawbacks indicated. Often two or more methods can be employed on different slides with advantage in order to bring out special features in the blood.

HÆMATOXYLIN.—Any good hæmatoxylin stain will stain most of the basic elements in the blood and most of the parasites. The number of preparations used is large. The formula recommended is composed of a mixture of—

Hæmatin	.. .. ..	2½ grammes.
Absolute alcohol	.. ..	50 cc.

Alum	50 grammes or to saturation.
Water	1000 cc.

The hæmatin is dissolved in the alcohol and added to the solution of alum in water.

In warm weather this stain matures rapidly, two or three weeks being sufficient. When mature the sides of the vessel containing the stain are deeply stained. Like all other hæmatoxylin stains, it must be tested before use to find the time required for staining. When properly mature this preparation requires about seven to ten minutes to stain blood well. It need not be filtered immediately before use. The stain may be placed over the film, or the slide with the film on it may be immersed in a pot of the stain, which should be well shaken before use.

If the stain is placed on the slide do not pour off the stain, but flush it off. If well flushed, even when a dirty stain is used, little deposit will be left on the film. If the stain be poured off, however much the slide is then flushed or washed, dirt from the stain will adhere to the film. After flushing off the stain leave in ordinary tap water for five minutes. Drain and allow to dry.

As a counter-stain eosine is useful. An aqueous 1 per cent. solution of yellow eosine (soluble in alcohol) is used. It will stain in twenty to thirty seconds; then wash and allow to dry. A film so prepared is in a fit condition for examination with an oil immersion lens. The oil can be placed directly on the films, but if it is intended to keep the film, it is simpler to mount in xylol balsam and then examine. Plate I. shows the appearances of the blood-cells stained in this manner.

Red Corpuscles.—The red corpuscles are stained by the eosine, and the depth of the colour varies according to the richness in hæmoglobin of the corpuscles. As in fresh blood, the size, depth of colour and shape of the red corpuscle should be observed. Among the rarer

forms of normal blood are red corpuscles which hardly stain with eosine, shadow or ghost corpuscles, polychromatic corpuscles, which are faintly stained with both stains, so as to have a purplish colour (Plate I., 10), and red corpuscles containing granules which stain deeply with the basic stain used, hæmatoxylin. The last cells are described as containing basophilic granules (Plate I., 9). Nucleated red corpuscles are very rarely present in the blood of healthy individuals, but are found not only in blood of patients markedly anæmic but also in some cases of malaria, &c. The nucleated red corpuscles have a nucleus staining deeply, but not evenly, with hæmatoxylin (Plate I., 3 and 4). They have a sharply defined margin. Not unfrequently the nucleated red corpuscle itself is polychromatic, or contains basophilic granules. Such nucleated red corpuscles may be larger (megaloblasts), smaller (microblasts), or the same size (normoblasts), as the normal.

The Blood Platelets are stained feebly with both stains and have a uniform faint purple colour. In an overstained specimen the network of fibrin filaments, starting from either a single plate or a group, is plainly brought out, but in a normally stained specimen only the platelets and the bases of these filaments are revealed (Plate I., 5).

White Corpuscles.—The leucocytes have their nuclei stained deep blue. The protoplasm is stained differently in the different varieties of leucocytes, but granules, with the exception of those staining deeply with eosine, are not brought out by this method. They are visible in the unstained leucocytes and can be stained by other stains.

In normal blood four varieties of white corpuscles can be differentiated. Of these two have a single more or less rounded nucleus. These mononuclear leucocytes are of two classes, though it is not always easy to say to which class a given mononuclear leucocyte belongs.

Still, with practice the number of doubtful instances greatly diminishes. The points to be considered in the differentiation are the size of the corpuscle, the shape and staining reactions of the nucleus, the stain taken up by the protoplasm, and the relative amount of protoplasm as compared with the nucleus.

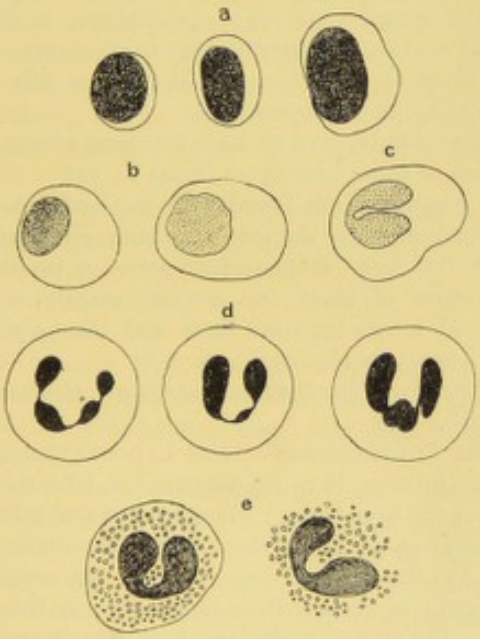


FIG. 23.
a, Lymphocytes; *b*, large mononuclear leucocytes; *c*, transitional leucocyte; *d*, polymorphonuclear leucocytes; *e*, eosinophile leucocytes.

(1) The small mononuclear leucocyte, or LYMPHOCYTE, is usually not much larger than a red corpuscle and varies from 7 μ to 12 μ. The nucleus stains deeply and forms the greater part of the corpuscle. The protoplasm is often reduced to a mere rim, and in any case, is relatively scanty in proportion to the size of the nucleus (fig. 23*a*).

The protoplasm is stained faintly pink, much the same as the protoplasm in the polymorphonuclear leucocyte (Plate I., 11).

(2) The Large Mononuclear Leucocytes (fig. 23*b*), sometimes called the hyaline cells, are variable in size, but some of them form the largest white elements in normal blood. The nucleus is not so deeply stained as in the lymphocyte. The protoplasm is relatively abundant and stains slightly with basic stains. It may be unstained or faint blue, or, if pink, is less so than the polymorphonuclear leucocyte.

All these points have to be taken into account in the separation of these leucocytes (Plate I., 12).

Some corpuscles are found with the nuclei deeply indented, or horse-shoe shaped. In staining reactions they resemble the large mononuclear and are probably advanced forms of these and not, as usually described, transitional fo ms between these and the polymorphonuclear leucocytes (fig. 23*c*).

The other two classes of leucocytes are much easier to distinguish.

(3) The Polymorphonuclear Leucocytes (fig. 23*d*), sometimes incorrectly called polynuclear, form the greater number of the leucocytes. They are rounded cells, which are granular in the fresh blood, but the granules are not stained by the method we are now discussing. The characteristic of these cells is the variety in form of the nucleus. The nucleus stains deeply with the hæmatoxylin and at first sight appears to be multiple. Closer examination shows that the different parts of the nucleus are really connected together, though often by a mere string or filament.

The form in dried uncompressed specimens is round, the size fairly uniform, and the protoplasm stains a faint pink (Plate I., 13).

(4) The fourth variety has a deeply indented nucleus, sometimes divided into three. The nucleus does not

stain so deeply with hæmatoxylin as in the polymorphonuclear leucocytes, but the characteristic of this leucocyte is the presence of a large number of coarse granules which stain deeply with eosine. Hence these leucocytes are called *eosinophile* (fig. 23*e*). This leucocyte is more loosely attached together than any other, and it is no uncommon event for one to be ruptured in making the film, so that the nucleus is seen surrounded by a cloud of granules stained with eosine (Plate I., 14).

These four varieties of leucocytes are all present in normal blood, but in relative numbers varying within comparatively small limits. The variations are shown in fig. 23.

The normal proportions are given variously as :—

Lymphocytes	10-25	per cent.
Large mononuclear	5-10	,, ,,
Polymorphonuclear	65-75	,, ,,
Eosinophiles	2-4	,, ,,

It will be seen that the lymphocytes are the most variable elements and, in an individual, they vary during the same day from hour to hour, according to the stage of digestion.

In many diseases, and for some time after these diseases, there is a marked variation in the relative proportions of these blood elements. A most important variation is that which occurs during, and still more markedly after, a malarial attack. The leucocytic variation, characteristic of malaria, is a relative increase in the large mononuclear elements, so that they constitute 20 per cent. or more of the leucocytes found. The increase appears to be constant and it is rarely less than 20 per cent., though it may be twice as great, so that they may in that case constitute 40 per cent. or even more of the total leucocytes.

It occurs in all forms of malaria and persists after all other signs or symptoms of malaria have disappeared. It is found sometimes three months or more after an

attack and rarely disappears, or even diminishes, in a month. It is not affected by quinine. It is occasionally, but rarely, found in typhoid and Malta fever and in other conditions, but in these diseases it is not persistent. In trypanosomiasis it appears to be constant. The total number of leucocytes is not increased, and there is a decrease in the total number of leucocytes in malaria during the febrile period.

A relative increase in the polymorphonuclear elements occurs in pneumonia and in many septic conditions, particularly when deep-seated abscesses form, such as in perityphlitic or hepatic abscess. In such cases there is also an absolute increase in the number of leucocytes. These diseases can therefore be distinguished from malaria, which they may resemble clinically, by a differential leucocyte count.

Increase in the relative proportion of eosinophiles occurs from many causes, some of which are unknown. It is marked in most cases of anæmia from ankylostomiasis and occurs also in many cases of filariasis and in some cases of bilharzia infection, and is said to be constant in trichinosis. The blood examination may often give a hint as to the presence of some of these parasites. As the increase occurs also from unknown causes, and in some skin diseases, in itself it is of no certain diagnostic value.

The proportion of lymphocytes is so variable that only an enormous increase is of importance. This occurs in scurvy and is associated with an increase in the other mononuclear elements.

A differential leucocyte count must not be confused with an actual enumeration of the number of leucocytes present in a given volume of blood. That has to be determined separately as we shall see subsequently. To make a differential count of the leucocytes a dried film of a small but uncertain volume of blood is prepared and

stained. All the leucocytes found in a systematic examination of a part of this film are counted and the percentage of each different variety met with is thus ascertained. For accurate work not less than 500 should be counted, but for clinical purposes 200 will often suffice. The edges of the film where leucocytes are most numerous, should not be included in the enumeration.

Abnormal elements resembling leucocytes are present in certain diseases, particularly in leucocythæmia. These abnormal elements are known as MYELOCYTES from their similarity to cells found normally in the bone marrow (fig. 24). They are of three kinds, all mononuclear.

FIG. 24.

(1) The first form is variable in size, the greater number of them are much larger than the large mononuclear leucocytes. With eosine and hæmatoxylin, as the granules which they contain are not stained, it is sometimes difficult to distinguish the smaller ones from the larger of the mononuclear leucocytes. For practical purposes the difficulty is unimportant, as when myelocytes occur they are common and most of them are readily distinguished from the leucocytes.

In these myelocytes the nucleus stains less readily and is therefore paler than that of the large mononuclear leucocytes. The edge of the nucleus is frequently ragged. The protoplasm is abundant and stains in many cases more deeply with eosine than the large mononuclear leucocytes do. Mitotic figures are often met with.

(2 and 3) The other two forms of myelocytes contain

granules which stain deeply with eosine. They are subdivided according to the size of these granules, which may be coarse, as in the eosinophile leucocytes, or fine. The distinction is probably unimportant. These myelocytes are distinguished at once from eosinophile leucocytes by the single nucleus, and from each other by the size of the eosinophile granules (Plate I., 15, 16, 17).

These cells are the abnormal cellular constituents that may be met with in blood specimens stained with eosine and hæmatoxylin, and they must be clearly recognised before any satisfactory examination for parasites can be made. In themselves they are of considerable importance in the recognition of various diseases and for prognosis.

In pernicious anæmia and in chlorosis the changes in the red corpuscles, the irregularity in their size, shape, and colouring are of clinical value, and in most tropical anæmias, including that occurring in malaria, the changes are similar to those in a mild case of pernicious anæmia.*

Leucocythæmia is readily recognised by the enormous increase in the number of the white elements which, as we have seen, take on basic stains. This increase is so great that the appearance of a dried film indicates it unmistakably, and it is not, for diagnostic purposes, necessary to make any count. The presence in numbers of the eosinophile myelocytes is conclusive proof of the implication of the bone marrow, whilst the absence of this form of abnormal cell indicates more probably a lymphatic leucocythæmia.

* Between these two forms of anæmia the main difference observed in blood examination is that in chlorosis the number is not diminished, but the hæmoglobin is, so that each corpuscle is poor in hæmoglobin. In pernicious anæmia there is a great diminution in the number of corpuscles, but the hæmoglobin value of the corpuscles averages much the same as normal blood. Mixed or intermediate cases occur.

In all forms of leucocythæmia decided changes are also found in the red corpuscles. Irregularities in size, shape and depth of colour are common, and nucleated red blood corpuscles occur often in large numbers. Polychromatic red corpuscles and red corpuscles showing basophilic granules are also common.

If no abnormal cells are present the relative proportions of the normal cells may be so changed that we can diagnose with some degree of probability septic processes, recent malaria or helminthiasis.

Eosine and hæmatoxylin can be used for staining any of the parasites found in blood. The stains are easily prepared, keep well, and their use is not dependent on distilled water or appliances which are not obtainable everywhere.

It is not a very brilliant stain, and therefore other stains giving more marked contrast are for some purposes preferable. It does not stain the granules present in many of the white elements of the blood, and though of general application, other stains are of greater value for special purposes and have special advantages.

Two combinations of methylene blue and eosine dissolved in methyl alcohol are much used. The first is the Louis Jenner stain. It is made by adding an eosine aqueous solution to one of methylene blue. The stains combine and form a precipitate which is collected in a filter, dried and dissolved in methylic alcohol.

This stain can only be used with films that have *not* been fixed. The methylic alcohol does all the fixing required. Films fixed in methylic alcohol do not stain satisfactorily. Distilled water is also an essential.

The stain may be placed on the film, slide or cover-glass for three and a half to four minutes, or, and this is better, the slide or cover-glass can be placed in the stain in a well-stoppered bottle for the same length of time. The time must be kept accurately, carelessness in this

respect leading to poor results. The stain must be flushed off with distilled water, and it is better to allow the distilled water to stand on the film for half a minute after washing. The water can then be drained or blotted off, the film allowed to dry, and the specimen examined directly with the oil immersion. When it is considered desirable to keep the specimen a drop of xylol balsam should be placed on the film and covered with a cover-glass.

With this stain the red blood corpuscles are stained pink, the depth of colour varying with the amount of hæmoglobin which the corpuscles contain (Plate III., 1).

The nuclei of the leucocytes are stained a clear blue, the eosinophile granules are stained deep red, and the granules in the polymorphonuclear leucocytes, which it will be remembered are not stained with eosine and hæmatoxylin, are brought out as fine dull-reddish granules.

Basophilic granules contained in cells are stained blue, this occurs both in white cells and in some red corpuscles. This stain is a good stain for many parasites, particularly those of malaria. Bacilli and cocci are also stained blue.

Some specimens of the Louis Jenner stain bring out the special stain of the important constituent of nuclei known as *chromatin*. A modification of the method and of the methylene blue is required to bring out the chromatin with certainty.

Many methods have been employed for this purpose, most of them modifications of Romanowsky's method. The simplest, the most rapid, and on the whole most satisfactory of these methods is that introduced by Leishman. A saturated solution of methylene blue, preferably "Höchst's pure medicinal," is made. This solution has to be rendered polychrome, so that in addition to the pure blue colour of the ordinary methylene blue it is in part changed into a red stain. The change is indicated by a change in colour of the solution, so

that in thin layers it has a reddish tinge. The solution of methylene blue becomes to some extent polychrome when exposed to air for some months, but for practical purposes a quicker transformation is required. There are many methods. Repeated heating in a steriliser accelerates the change. Leishman uses a 1 per cent. solution of methylene blue (Grübler's), and adds 5 per cent. sodium carbonate to it. This solution he keeps at a temperature of 65° C. for twelve hours, and then exposes to air for a week or more.

J. H. Wright adds to a ½ per cent. solution of sodium bicarbonate 1 per cent. of methylene blue (Grübler's B. X., Koch's or Ehrlich's rectified). This solution is steamed in a steam steriliser for one hour, this effects the required transformation, and the solution may be used as soon as it is cold without filtering.

A convenient method, and more suitable where steam sterilisers are not available, is to treat the saturated solution of methylene blue with freshly precipitated oxide of silver. A solution of sodium hydrate is added to a solution of nitrate of silver till no more precipitate forms. The precipitate is washed till the washings are neutral to litmus paper. The precipitate, oxide of silver, is added to the saturated solution of methylene blue, and it is allowed to stand for twenty-four hours or more. A considerable proportion of the methylene blue in solution will be converted into polychrome methylene blue. The superjacent solution should be decanted off from the precipitated silver salts and filtered before use. It improves with keeping.

Whatever method be adopted for rendering the methylene blue polychrome the subsequent proceedings are the same.

One hundred cc. of this solution of polychrome methylene blue are placed in a large shallow vessel (a half-inch photographic tray is a suitable one), and then a

1 in 1,000 eosine aqueous solution is added till a thick film forms on the surface and the fluid shows the colour of the eosine. About 400 cc. or a little more will be required, but the change in colour is the guide. The mixture should be well stirred and then be allowed to stand exposed freely to air for some hours, stirring occasionally, and filtered. The residue in the filter is composed of the stain. It should be well washed with distilled water till the washing has only a faint bluish tinge; and then it is thoroughly dried, preferably in an incubator at blood heat. The stain must be finely powdered before use.

Two centigrammes of the powder can then be dissolved in 100 cc. of pure methyl alcohol, and the stain is ready for use. It is perhaps more convenient to make a saturated solution of the stain in methyl alcohol, filter in the cold, and dilute with one-tenth of its bulk of methyl alcohol, so that a solution is made which is not quite saturated.

To Use the Stain.—With a pipette two or three drops of the stain are placed on the dried *unfixed* blood film on slide or cover-glass, and allowed to stand on it for half to one minute. If it shows any tendency to dry over any parts of the film in this period fresh stain must be added. To the fluid stain on the slide at the expiration of this half or one minute distilled water must be added drop by drop, and by oscillating the slide the stain and water are mixed as rapidly as possible. The amount of water required should be about double that of stain, but a better guide is to add the water in such an amount that when mixed with the stain the dark blue colour of the latter is replaced by a pinkish colour in the mixture, whilst the precipitated stain can be seen floating in the fluid. With a little practice the right amount of water required in each case is easily found, and slight variations from exactitude are not

of great importance. The water mixed with the stain should be allowed to remain on the film for five minutes, or with old or thick films for a longer period. It is quite easy to watch the staining under a low power on the microscope.

The stain is then flushed off with distilled water, and a drop of distilled water is allowed to remain on the film for about one minute. A certain amount of the blue is dissolved, and the red corpuscles acquire a clearer red colour. This clearing with distilled water is essential to obtain good results. The more deeply the specimen is stained the longer will be the time required for clearing. This stage of the process is watched under the microscope and stopped when the clearing is sufficient. The water is then washed off rapidly with distilled water, the specimen drained or blotted and allowed to dry. Mount in xylol balsam and examine.

The principle of all modifications of the Romanowsky stain for chromatin is that the staining takes place during the precipitation of the stain; in the original processes during the precipitation of the mixture of aqueous solutions of the stains, and in Leishman's method during the precipitation by water of the combined stains dissolved in methyl alcohol.

Absolute alcohol with 2 per cent. aniline oil can be used as the solvent instead of methyl alcohol, and the solution treated as Leishman treats the methyl alcohol solution. The results are not so good as with methyl alcohol for a solvent, but are very fair. It is not to be recommended except where methyl alcohol cannot be obtained: the time for all the stages of the process should be doubled if this solvent be employed.

Another modification can be used for films fixed in alcohol.

In this method staining takes place during the admixture and mutual precipitation of the eosine and

polychrome methylene blue. A 1 per cent. solution of pure medicinal methylene blue (Grübler's) is made in distilled water and ½ per cent. sodium carbonate added. This solution keeps well and is fit for use when a reddish tinge appears. This change is expedited by keeping in an incubator.

A second stock solution is a 1 in 1,000 solution of eosine extra B.A. (Grübler). This is fit for use at once, and keeps well if not exposed to light.

These are stock solutions, and should be diluted with twenty-four parts of pure water before use. The solutions are rapidly mixed and stirred, and the slips or covers are placed with the film side downward in the mixture. The dish should be rocked from time to time and the films left in the stains for half an hour or more till well soaked. This is tested by examination of the slide whilst still wet under a low power.

The specimen should then be washed in distilled water, rapidly dried, and examined again under a low power. If too deeply stained a little distilled water may be left on the slide to clear it for a minute or more. Blot off the water, dry in the air and examine directly, or after mounting in Canada balsam.

In specimens of blood stained by Romanowsky's method and its modifications, there are several distinct colours to be observed (Plates III. and IV.).

Chromatin is stained red. Other elements taking basic stains are mostly stained blue in various shades, and the red corpuscles are stained a peculiar pale pink with the eosine. Some granules, as those in mast cells, are said to be metachromatic, as, though they stain deeply, the colour is different to that of the stain used. (Plate III., 7).

Polychromatic red corpuscles are stained purple and basophilic granules are well brought out as blue dots. The nuclei of nucleated red blood corpuscles are found

to be rich in chromatin, and consequently the nuclei are stained a deep violet-purple.

In corpuscles invaded by certain parasites, viz., those of human benign tertian, granules or dots staining red are found. In amphibian blood corpuscles invaded by one species of drepanidium similar granules occur. These granules are known as Schüffner's dots. and indicate a peculiar form of degeneration (Plate III., 21, and Plate IV., 7, 8, 9).

THE BLOOD PLATELETS are stained faint blue, with numerous red particles which sometimes form a mesh-work. These particles are deeply stained and render the platelets very conspicuous (Plate III., 2).

THE LEUCOCYTES, with the exception of the eosinophile, are well stained, but in many specimens the eosinophile granules do not show a clear red. The large size of the granules is shown, and there is no real difficulty even in a badly-stained specimen in recognising these elements. They do not, however, form as conspicuous objects as in specimens stained by Louis Jenner's stain.

The nuclei of the polymorphonuclear leucocytes stain purple. The staining is not regular but in patches. The protoplasm contains minute granules, usually in very large numbers, staining brownish-red. The protoplasm itself is very faintly stained.

THE LARGE MONONUCLEAR LEUCOCYTES.—The nuclei stain faintly purple. The staining is not uniform, but presents a thin grained appearance. The protoplasm stains a faint blue, and imbedded in it are granules, which may be coarse or fine, and stain a deep clear red (Plate III., 4).

THE LYMPHOCYTES.—The nuclei stain a deep purple from the large amount of chromatin contained. The staining is more uniform than in most of the leucocytes. The protoplasm is stained deep blue, is nearly uniform, and has no granules staining a different colour (Plate III., 3).

MAST CELLS.—The nuclei are stained very faintly, and when the basophilic granules are numerous are difficult to make out. The granules in the protoplasm form large and irregular masses, and stain a deep purple-brown (metachromatic) (Plate III., 7).

MYELOCYTES have in most cases a rather feebly-staining nucleus, poor in chromatin. The nuclei are large, but the relative amount of protoplasm varies greatly, in many cases a mere rim only of protoplasm is found (Plate III., 9).

Granules taking either the acid or basic stain, or both, are present in most of the cells, sometimes in small, but more commonly in large numbers. Sometimes two, or even three, classes of granules are present in the one cell (Plate III., 10 to 13).

A detailed classification of these abnormal cells would be very difficult, as intermediate forms abound.

Amongst these cells are a small number with a large nucleus richer in chromatin than most of the myelocytes, and a rim of protoplasm staining a deep blue. These are not unlike the large cells found in a case of trypanosomiasis and in other ill-determined blood conditions, but in those the protoplasm is relatively to the nucleus in larger amount (Plate III., 9).

The true myelocytes include the eosinophile myelocytes, but in these there is, in most cases, some admixture of neutrophile or basophile granules, as shown by this stain.

The main classes of granules revealed by this stain are pure oxyphile or eosinophile, basophile staining blue, and neutrophile, which take up both acid and basic stains, including in some instances the red modification of the methylene blue and metachromatic granules. According to the relative proportions of the stains, these granules present a range of colours from blue to red, or to a purple-brown, and differ also in the intensity of

the staining and the size of the granules. Too little is known of the micro-chemistry of such cells, or in detail of their origin, for the meaning and value of the different granules found to be of much practical importance at present.

CHAPTER IV.

ANIMAL PARASITES FOUND IN BLOOD.—Of the four great divisions of the protozoa, the sporozoa and mastigophora are found in human blood.

To the SPOROZOA belong the parasites which cause malaria in men. These are found in the red blood corpuscles.

The MASTIGOPHORA (flagellated organisms) are represented by trypanosomes, which are found in the blood plasma.*

Protozoa are found in the blood of many of the lower animals, and the better known of these will be considered in brief.

Belonging to the higher animal kingdom are TREMATODES, of which the *Bilharzia hæmatobia* is found in certain blood-vessels, and NEMATODES, represented by the filaria and filarial embryos.

THE EXAMINATION OF THE BLOOD FOR PROTOZOA.—An essential feature of the examination consists in the examination of the fluid blood as soon as possible after its removal from the body. Many of the parasites exist in the red blood corpuscles, so that the film must be so thin that in a great part of the film the red corpuscles are all lying flat and separate from each other.

* *Spirillum Obermeyeri*, the cause of relapsing fever, is by some believed to belong to the protozoa. It occurs in the blood plasma, and for convenience will be considered with this class of parasites.

The method of making such thin fluid films already described must be strictly adhered to. It is often urged that examinations of stained films are more convenient and better, but it cannot be too strongly insisted on that most of the important errors which have occurred have been due to the exclusive use of stained specimens, and also that the phenomena of life can only be satisfactorily observed in the fluid blood. These include some points of diagnostic value, namely, the character and movements of the pigment, and the activity of the amœboid movement and the formation of flagella.

Stained films have their value, and show more clearly some points in the structure of the parasites. In busy practice it is often more convenient to defer for some hours the examination of the films, and therefore stained specimens are in such cases more useful. In any case of difficulty, or when dealing with a parasite believed to be new, both methods should be adopted.

Dried films can be made by any of the methods already described, and the parasites stained by the methods recommended. The films deteriorate when kept, and should therefore be examined as early as convenient, though a delay of a few days is not of much importance.

Other methods can be adopted if only the presence or absence of parasites has to be determined.

The methods generally used can be divided into three :—

A.—Where preliminary fixation is required before staining.

B.—When fixation and staining are effected together.

C.—When fixation is avoided.

A.—Films to be fixed by immersion in alcohol or alcohol and ether.

(1) HÆMATOXYLIN alone, or HÆMATOXYLIN and EOSINE. These stains can be used as already described, but better results are obtained by doubling the time for staining with hæmatoxylin.

(2) Stain the fixed film with Borax Methylene Blue. This stain is composed of methylene blue 2 grammes, borax 5 grammes, and water 100 cc.

Place a few drops of the stain on the dried and fixed film and leave it for thirty seconds. Wash well with water, allow to dry, and examine directly, or mount in xylol balsam.

It is very easy to overstain by this method, and in such a case the red corpuscle will also be stained blue and the parasites will not stand out clearly.

This stain is the quickest used, and on account of the risk of over-staining some authorities dilute it with one, two, or three times the volume of water. The stain keeps well.

(3) Carbol Thionin.—A stock solution of thionin 1½ grammes, alcohol 10 cc., and 1 in 20 aqueous carbolic solution to 100 cc. is made. This stock solution keeps well, but is too strong to use for films. Before use it should be diluted with three parts of water. This diluted solution does not keep for more than a few days. For use, cover the film and leave the stain on for five minutes or more. It does not easily over-stain, so that only the minimum time need be remembered; still, to get good results half an hour is about the limit. Flush off the stain, allow to dry, and examine directly, or mount in Canada balsam.

It is a good, clear, transparent basic stain and gives a very fair contrast. Bacteria, as well as animal parasites, are well stained, and it is one of our best stains for the demonstration of parasites in tissues.

(4) Toluidine Blue is a stain which has some points of resemblance with thionin. The stain is best kept as a saturated alcoholic solution and diluted for use with twenty parts of 1 in 80 aqueous solution of carbolic acid. The fixed film should be covered with the stain for ten minutes or more. It is difficult to over-

stain, and good results are obtained even if the film be left in the stain for twenty-four hours. This is an advantage, as the specimens can be left to stain whilst other occupations are pursued. The main advantage of the stain is that the pigment is less obscured than in specimens stained by carbol thionin.

If blood examinations are frequently required, it is well to keep the stains in a wide-necked stoppered

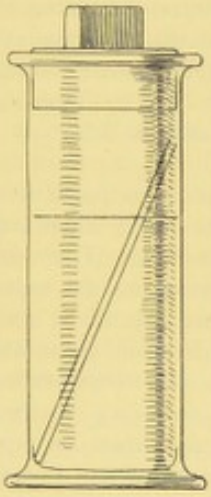


Fig. 25.

bottle, and simply place the slide in the stain for the time required instead of putting the stain on the slide. Many of the stains form films on the surface, and if this film of stain comes into contact with the blood film it will adhere to it. The bottle of stain should be shaken vigorously before use. The fixing agent can be kept in the same way (fig. 25).

B.—These methods include the use of Louis Jenner's stain and the stain used in Leishman's modification

of Romanowsky's method, as in both of these the methyl alcohol fixes the film. The method of using these stains for the examination of normal blood has been already described. It suffices for the demonstration of all the protozoa. The stain, particularly Leishman's, gives most brilliant results, and show more points in the structure of parasites than any other method. The disadvantages are: (1) The necessity of having distilled water, though where the rainfall is heavy and away from the sea, rain-water can often be used; (2) methyl alcohol is very volatile; (3) the stains, under circumstances not thoroughly understood, seem to sometimes lose their strength in the Tropics, and consequently are not so universally reliable as the simpler stains first described.

Louis Jenner's stain in particular is unreliable, and seems to deteriorate either when kept in the solid condition or when dissolved. The usual failing is in the basic portion of the stain, and unless the nuclei of the leucocytes are stained a brilliant blue the stain is worthless for the demonstration of parasites.

Leishman's stain also deteriorates, but can be made satisfactorily with certainty in the manner described. But both these stains must be rejected if the normal constituents of the blood are not satisfactorily stained by them.

The results obtained by the use of these stains are so clear and good that it is a pity to discard their use, as a film can be much more rapidly examined when stained by Leishman's method than when stained by any other method. The worker must, however, be prepared to make up his own stain, and, if need be, to distil water before he is justified in trusting to these stains alone.

C.—When parasites are scanty they may be easily overlooked if thin films only are examined. Thick

films, if fixed, are too opaque for examination after fixation and staining.

A useful method with the larger parasites is to make a very thick film and allow it to dry. When dry place in water, the hæmoglobin will be dissolved out and only parasites, leucocytes, blood platelets and fibrin, with the decolourised remnants of the blood corpuscles, will be left. Such decolourised films, when dry, can be stained with any of the basic stains. There is usually considerable distortion of the parasites, but many of them, particularly crescents, are quite recognisable. Trypanosomes can be more readily found by this method than in thin films. A good stain to use with these decolourised films is carbol fuchsin, diluted with two parts of water. Ross prefers to decolourise the film with a weak aqueous solution of eosine, and counterstain with a weak solution of polychrome blue. It is a useful diagnostic method, but not suitable for obtaining good specimens of the more delicate parasites.

General Summary of the Development of the Hæmosporidia.

The Hæmosporidia are parasitic in their entire existence, and require for complete development two hosts: the one a warm-blooded animal, and the other usually an insect. In the warm-blooded host reproduction takes place asexually, by the breaking up of each organism into a number of young forms or spores.

Each of these spores enters a red corpuscle, and when it has reached its full development it, in turn, breaks up into spores. This is the ENDOGENOUS or ASEXUAL CYCLE OF DEVELOPMENT. The host, during this cycle, is the INTERMEDIATE HOST. The parasites which develop in this manner are known as SPOROCYTES.

Some of the spores, however, instead of forming sporocytes, assume the sexual or GAMETE form. These do not

reproduce, or undergo any further change, whilst in the intermediate host. If they are taken up by the definitive host they become sexually active, conjugation takes place, and further development follows. The product of the conjugation, the fertilised female, increases in size and forms a cyst. The contents of this cyst

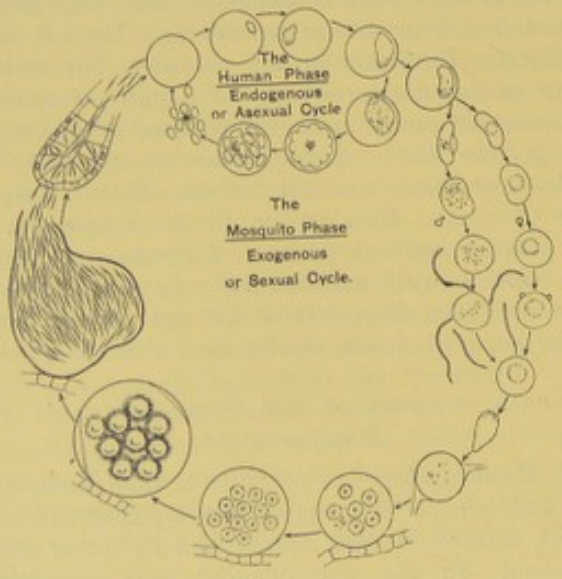


FIG. 26.

divides into several masses, blastophores, from which small, thread-like bodies, zygotoblasts or sporozoites, are formed. These bodies, when introduced into a suitable animal—the intermediate host—become sporocytes.

This cycle is a sexual one, and the host during this period is therefore the DEFINITIVE HOST. Mosquitoes belonging to several genera of the *Anophelina* are the

definitive hosts of the parasites of malaria, whilst man is the only known intermediate host.

The diagram (fig. 26) represents in a graphic form the two methods of reproduction. The smaller circle represents the asexual cycle of reproduction, and the larger the conjugation of the male and female sexual forms, with the further development of the fertilised female.

Parasites of Malaria.—As seen in fresh living blood the youngest form of malaria parasite is a small, white, rounded body in or on a red corpuscle. A clearer portion can sometimes be made out inside it. At this stage it varies, according to the species of the parasite, from one-eighth to one-quarter the diameter of the red blood corpuscle. Even the youngest forms of the parasite often show at the edge some sign of amœboid movement. The parasite increases in size, and the amœboid movements become pronounced. A parasite seen a few hours later will be observed to be not only larger, but to have a few grains of pigment scattered about inside it. The colour of the pigment and the size of the grains varies with the species of the parasite. When the parasite has reached the fullest growth its pigment commences to aggregate, usually towards the centre, and traces of division appear in the surrounding protoplasm. These traces of division become obvious, and soon the central aggregation of pigment can be seen to be surrounded by separate rounded masses of protoplasm—the spores. The remnant of the red corpuscle then gives way and the spores are poured out into the plasma. A mature parasite, if kept under observation on a warm stage, can frequently be seen to break up in this manner.

In many cases a leucocyte will appear in the field and devour the pigment, and often some of the spores. That a large number of spores are destroyed is shown by the fact that though each tertian parasite forms

twenty or more spores, the number of parasites in each successive cycle does not as a rule increase.

Free spores are not found in the circulating blood. Apparently they either rapidly take shelter in a red blood corpuscle or are destroyed by leucocytes or in some other manner.

Stained specimens demonstrate some further points in the structure of the parasites. The youngest form, the amœbula, is shown to consist of a ring of protoplasm staining with basic stains, a clear unstained space, the vesicular nucleus, and a deeply-stained spot, or nucleolus, usually in contact with the ring of protoplasm.

This is the type of the young form of all the hæmosporidia. They are all composed of a nucleolus staining deeply, a "vesicular" nucleus, which does not stain with either acid or basic stains, and a surrounding protoplasm which stains, but much less intensely than the nucleolus, with basic stains. It is only this surrounding protoplasm which is amœboid, and consequently in the very young forms amœboid movement is not very great, as this protoplasm is so scanty.

The increased growth of the parasite is mainly due to the increased growth of the ring of protoplasm, though the vesicular nucleus also enlarges. Pigment or the residue of the digested hæmoglobin is deposited in this protoplasm only. With further growth the vesicular nucleus breaks up and disappears, and all that is seen is an irregularly-stained parasite with pigment scattered through it. Later the pigment becomes pushed into one block, and the surrounding protoplasm is seen to be divided into masses, each with a deeply-stained spot, the nucleolus of the young spore.

Beyond showing the "ring form," none of the following stains, hæmatoxylin, thionin, methyl blue, &c., disclose any further structural changes beyond those seen in fresh blood.

Romanowsky's method, or, better, Leishman's modification of this method, shows more markedly the structural changes, and, in particular, the varying arrangement of the chromatin. With this stain the youngest form, amœbula or ring form, is shown to have the chromatin arranged as a solid block — the nucleolus — which is stained deep red. The ring of protoplasm stains blue, whilst the vesicular nucleus is unstained. At a later stage the chromatin, instead of being in a solid mass, is seen to be composed of scattered points, arranged at part of the periphery of the vesicular nucleus. Still later when the vesicular nucleus disappears, points of chromatin are found diffused through the protoplasm. This is called "fragmentation" of the nucleolus. Still later the chromatin aggregates into small masses towards the periphery, and a secondary division of this mass takes place, resulting in the formation of a number of small chromatin nodules, the nucleoli of the young spores.

When sporulation is complete each of these chromatin nodules is situated in the interior of a portion of the protoplasm of the parasite, and so forms the spore. The pigment takes no part in the process, and with a small residual portion of the protoplasm of the parasite is pushed into a mass, usually towards the centre of the group of spores. When the corpuscle bursts and the spores are liberated, the pigment is devoured by leucocytes, usually the large mononuclear leucocytes, and thus the "pigmented leucocytes" are formed.

The chromatin in the parasites destined to become *gametocytes* undergoes different changes. The first stage is the same as in the young parasite, which will ultimately divide asexually into spores—the sporocyte. The chromatin in the young or "ring" form of the gametocyte, as of the sporocyte, is arranged in a solid block. This chromatin subsequently divides into separate granules,

but does not become suffused throughout the protoplasm as it does in the sporocyte. In the full-grown gametocyte the chromatin, composed of numerous particles packed together, forms one mass in the interior of the parasite, surrounded by a zone free from pigment and staining feebly. The changes in the arrangement of the chromatin after the blood is shed and the gametocytes become sexually active will be considered with the sexual or mosquito phase of the existence of the malaria parasite.

All the human malaria parasites, the similar parasites in other mammalia and birds, as far as is known, conform to this general type, though some digest the hæmoglobin completely without forming pigment.

The distinctive points on which the division of the human parasites into distinct species is made are as follows :—

(1) Duration of the asexual cycle.
(2) Number of spores formed at each sporulation.
(3) Activity of movement.
(4) Preferential sites for sporulation.
(5) Differences in digestive processes in different parasites as indicated by the differences in pigment.
(6) Effect of the parasite on the corpuscle which contains it.
(7) Shape and appearance of the gametocyte.

The methods of examination described are ample for determining these points.

(1) *The length of cycle* can be readily ascertained in the case of parasites which sporulate in the peripheral blood. This blood is examined at intervals, so as to determine the length of time between the sporulation of a group of the parasites and the steady growth of this group up to the next period of sporulation. In benign tertian and quartan this is readily done, and it will be found that the period or length of cycle is

approximately forty-eight and seventy-two hours respectively. It is difficult to determine in malignant tertian (æstivo-autumnal or sub-tertian) malaria, as only the young sporocytes and mature gametocytes are common in the peripheral blood. The period for this species is certainly variable, and the parasites are commonly in several stages of growth, so that periodicity is not so clearly defined as in the other species of parasites of human malaria.

(2) *The number of spores* can be counted in the fresh or stained blood when the parasites are fully mature. If stained for chromatin, the number of spores can be counted earlier. It will be found that in benign tertian the spores are usually about 20, but may be as low as 15 or as high as 25, or even more. In benign quartan 12 is a maximum rarely exceeded, whilst 8, 9, or 10 are the common numbers. The number in sub-tertian is more variable—4 to 30.

(3) *The activity* of the amœboid movement can only be determined with certainty in the living blood. Internal movement in the parasite itself is also shown in the fresh fluid blood by movement of the pigment in the parasite.

Amœboid movements can be inferred in stained specimens, as the parasites present great varieties in shape, and frequently where amœboid movements have been active, in the stained specimens the pseudopodia can still be seen.

(4) *The selective site* for sporulation is of great importance, as one species, the malignant tertian (sub-tertian) sporulates almost exclusively in the internal organs, and the occasional malignant clinical course of the disease caused by this parasite is due to the selection of the brain as a site for sporulation.

The absence of full-grown forms and the determination of the absence of sporulating forms indicates that the

parasites are sporulating elsewhere, *i.e.*, in the internal organs. *Post-mortem* examination of fatal cases shows in which organs the sporulating parasites are, but the symptoms often give a clue.

Benign tertian parasites sporulate to a considerable extent in the circulating blood, though the splenic sinuses are their preferential resort at this period. Quartan sporulates freely in the circulating blood, whilst subtertian (malignant tertian) is hardly ever found sporulating except in the visceral capillaries.

All the phases of benign tertian and quartan can be observed in the blood obtained by pricking the finger or ear, and therefore the determination of the length of the cycle with these parasites is easy. With malignant tertian, on the other hand, the stage of sporulation, and even the full-grown sporocytes, are rarely to be observed in the peripheral blood, and though the full-grown gametes are common in the blood, and we have no reason for supposing that the youngest forms are absent, the intermediate stages of growth cannot be found. Puncture of the spleen in the living subject may show these forms. If undertaken aseptically the operation is considered to be practically free from risk to the patient; but as accidents have occurred this method should not be employed except in cases where certainty of diagnosis is absolutely necessary.

In fatal cases with cerebral symptoms, the sporulating and full-grown forms can be observed in enormous numbers in the brain and often in other organs—lungs, suprarenals, liver, &c. In other fatal cases they may be found in greatest numbers in the intestinal mucosa, pancreas, kidneys, &c.

The organ in which the parasites are most commonly found, *post-mortem*, is the brain, and cerebral symptoms are common in so many cases that recover that it seems probable that this is a favourite site. It must be remem-

bered, however, that as the blocking of the cerebral capillaries is the most common cause of death in acute malaria, that the proportion of fatal cases with this complication gives an exaggerated idea of the frequency with which this site is selected by the parasites.

For diagnostic purposes it suffices to take a small portion of the fresh brain substance and squash it between the slide and cover-glass. The capillaries in a case of cerebral malaria will then be seen to be filled with grains of black pigment. Though the parasites themselves cannot be seen, these grains of pigment are diagnostic, as they are contained in the full-grown or sporulating parasite.

It is not absolutely necessary to open the skull, though it is better to do so. The needle of a large exploring syringe can be forced through the orbital plate of the frontal bone and the brain stirred up a little, suction with the syringe will then usually bring away sufficient brain matter for examination. As the puncture is made through the conjunctiva no disfigurement results, and the site of puncture will be covered by the eyelid.

The vessels on the *pia mater*, particularly at the base of the brain, are frequently pigmented. This pigmentation must not be confused with malarial pigmentation. The pigment is not contained, as it is in malaria, in the capillaries, but in their walls, and is insoluble in alkalies which readily dissolve melanin. The finely granular arrangement of this natural pigmentation differs from the coarser arrangement of the melanin particles, and the colour is brown not black. This pigmentation, non-malarial, occurs in all races, but is commoner in the coloured races. It is found in new- or still-born children whose organs are free from malarial pigmentation.

To demonstrate the arrangement of the pigment granules of malaria in a hardened brain, thick sections should be cut. These can be quite easily cut by hand,

and without any staining passed through absolute alcohol and then oil of cloves to dissolve the fatty brain constituents and render the section transparent. The section can then be mounted in balsam, and in a malarial case every capillary will then be seen to be mapped out by the contained pigment granules almost as if it had been injected.

These methods, though useful for rapid diagnosis, do not show the parasite. With the fresh brain specimens, whether a squashed fragment or a fragment drawn out with the exploring syringe be examined, parasites will often be seen in corpuscles which have escaped from the capillaries.

To show the parasites well it is necessary to stain them. With the fresh brain it is not necessary to cut sections nor is it advisable. A smear should be made of the brain substance, and this should be caused to dry rapidly by waving it in the air—not by the application of heat. The smear need not be very thin, as the greater part of the brain matter is subsequently dissolved. The smear can be stained by Leishman's method, but must then be thoroughly dried to dehydrate, and mounted in xylol balsam. This method shows the chromatin in the parasites, but the drying causes much distortion of the surrounding tissues.

If this method be not adopted, hæmatoxylin gives good and permanent results, and carbol thionin also gives very good results. The procedure is as follows: Fix the smear in alcohol for ten minutes and allow to dry.

To stain with hæmatoxylin, cover the smear with a hæmatein solution and leave for ten minutes. Flush off stain and place the slide in water for five minutes. Dehydrate with spirit and oil of cloves. Mount in xylol balsam.

With carbon thionin the procedure is rather more complicated and requires more care. It is, however,

a general method, and is a suitable one also for the demonstration of micro-organisms in tissues.

Fix the smear in absolute alcohol as before, and cover it with the strong carbol thionin solution. Leave for ten to fifteen minutes. It is essential that at this stage the specimen should be very much over-stained, as much stain is lost in the subsequent processes. Flush off the stain with water. Pass through methylated spirits, *not* absolute alcohol. Much stain will come away, and care must be exercised that the specimen is still over-stained when removed from the spirit. The time the specimen is left in the spirit is determined entirely by the colour. It cannot be completely dehydrated at this stage, or too much colour would be removed. Drain off and *gently* blot off excess of spirit. Cover with oil of cloves and place under the microscope. The oil of cloves will dissolve out the brain fatty matter, complete the dehydration, and slowly remove the excess of stain. When it is observed that nearly enough stain is removed, a cover-glass can be placed over the specimen and an examination made with an oil immersion lens. If the specimen is well stained the cover-glass can be removed, and the specimen placed in xylol to remove the oil of cloves, which would otherwise ultimately decolourise the specimen. Finally it is mounted in xylol Canada balsam.

Specimens of brain hardened in absolute alcohol can be used. Thin sections are required, imbedded in paraffin for choice. The processes are the same as for brain smears after the paraffin has been removed from the specimen by xylol, the xylol by alcohol, and the alcohol by water; but the section must not be allowed to dry at any stage.

The parasites in sections show well, but are smaller, only about half the size of those in the smears made from the fresh brain, as the fixative agent causes much

shrinking of the parasites (Plate II., 3*a*, 4*a*). As this parasite is the smallest of the human malaria parasites, and when full-grown often little more than half the diameter of the red blood corpuscles, there is in these shrunken specimens considerable difficulty in seeing the spores into which the parasites are broken up with the one-twelfth oil immersion.

In these specimens the corpuscle containing the parasite is not lying singly or flat, as it is in the blood film, but is one of the many corpuscles packed into the capillary, so that it is exceptional for the outline of the corpuscle containing the parasite to be made out.

In the large vessels parasites are not so common. In the small vessels the corpuscles containing the parasites are often found only in contact with the wall of the vessel, and no parasites are contained in the corpuscles towards the centre of the vessel. The largest number of the parasites are in the corpuscles in the capillaries.

This occurrence in the minute capillaries results in a blood stasis more or less complete. Such a stasis involving a large part of the brain, results in headache, drowsiness, and coma in adults, rarely delirium, and in convulsions and coma in young children, and is the most common cause of death in acute malaria. The process is often spoken of as *thrombosis*. This is incorrect; there is no coagulum formed, no fibrin, and the leucocytes are not aggregated in the capillaries and take no part in the process. Clinically, where active treatment is adopted we have abundant evidence that the condition is a transient one. Speedy and complete recovery from the condition of complete coma frequently takes place under energetic treatment with quinine.

The parasites themselves are usually at different stages. Quite young parasites, hardly larger than the spores, may be found. More commonly the great

majority of the parasites contain centralised pigment and have lost their vesicular nucleus. In some specimens a large proportion, in others a small proportion, will be found sporulating.

The number of spores varies greatly, and in some specimens only seven or eight spores will be found to each parasite. In other cases the number will be twenty or more.

This variation in the number of spores is one of the distinctions on which reliance has been placed for the subdivision of this species into three.

In most parts of the body a temporary partial stasis of the blood in the capillaries leads to no sudden fatal changes or symptoms, and consequently, unless the brain is also involved, a fatal result is not common. There are, however, peculiar risks attending another region—the intestines. Stasis occurring in the capillaries of the mucosa impairs the vitality of the cells and renders them liable to be invaded by some of the bacterial contents of the alimentary tract. Secondary inflammation and superficial necrosis may thus result, and so indirectly by lowering the nutrition of the mucosa a fatal enteritis may be set up. There is some reason for believing that sufficient attention has not been paid to the indirect results of repeated blood stasis in the various viscera consequent on malarial infection.

The parasites in these and other situations are best demonstrated in sections stained with hæmatoxylin, or carbol thionin, as already described for the brain. Carbol thionin has the additional advantage of staining the micro-organisms which have invaded the mucosa. These, however, can be shown in separate specimens somewhat better, particularly those micro-organisms which retain their stain when treated by Gram's method.

(5) *Melanin*, malarial pigment, or simply "pigment," is the residue from the digestion of hæmoglobin, and

contains the excess of iron over the minute amount required by the parasite.

In the different species it is deposited in different forms. In the quartan it is deposited as granules, which are coarse and black, and in the benign tertian the colour varies from a yellow-brown to a dark brown, but is always in fine granules: in the sub-tertian the

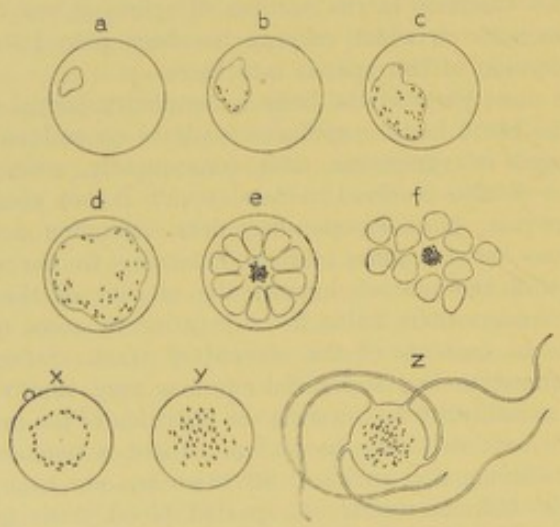


FIG. 27.

a—f, Phases in the asexual development of the quartan parasite; *x—z*, phases in the sexual development.

pigment is not commonly seen in the early forms present in the peripheral blood. When it is found it is in fine, black granules, which aggregate into a mass earlier than in the other forms of parasites.*

* The hæmosporidia of cattle, horses and dogs do not form pigment.

(6) The parasite affects the corpuscle containing it in different ways.

In the quartan fever, although the parasite is in the interior of the corpuscle, the bulk, or at any rate the diameter of a corpuscle containing the parasite, is, in

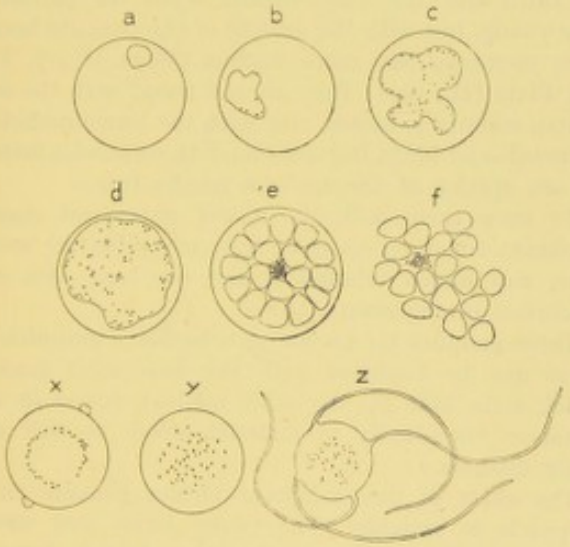


FIG. 28.

a—f, Phases in the asexual development of the benign tertian parasite; *x—z*, phases in the sexual development.

the majority of instances, slightly below the average. The colour of the red corpuscle is not lighter and is frequently a trifle darker than the average of the red corpuscles.

In benign tertian there is a great difference, as the diameter of the corpuscle is decidedly above the average and the corpuscle is pale. This is well seen both in stained and unstained specimens. The corpuscle is

easily distorted, and is consequently frequently compressed by neighbouring blood cells when these are in contact with it. Leishman's and some of the other similar stains reveal still further changes, as granules staining deeply red are found throughout the corpuscle in parts not invaded by the parasites. These granules are small and not very distinct when the parasite is very young, but with the growth of the parasite become more numerous and more distinct (Plate IV., 7, 8, 9, and Plate III., 21). They are not found with the other human malaria parasites, nor with the hæmamœbidæ of mammals and birds, but are found in corpuscles infected by one species of drepanidium in the frog.

To show them well, the mixed water and stain in Leishman's method must be left on double the normal time, and fifteen minutes is not too long with most specimens of the stain.

These granules are known as Schüffner's granules, and must not be confused with the basophilic granules, which stain blue by the same method, found in some instances abundantly in malarial blood, as well as in other diseases.

The effect of the malignant tertian parasite on the corpuscle is variable. The young forms met with in peripheral blood do not appreciably affect it, but occasionally the corpuscle is changed in colour to a yellowish or brassy tinge. With the more advanced stages of the parasite the corpuscle, without increasing in size, becomes more or less decolourised. In the full-grown parasite, whether sporocyte or gametocyte, the remnant of the red corpuscle is a mere colourless shadow, which is not always easy to define (Plate II., 1 to 8).

(7) The shape of the gametocyte in benign tertian and quartan is that of a rounded body difficult to distinguish from the full-grown sporocyte before sporulation has taken place. In malignant tertian the gametocyte

is the so-called "crescent." It is better described as sausage-shaped. It is not truly crescent-shaped, as, though often slightly curved, the two ends are broad

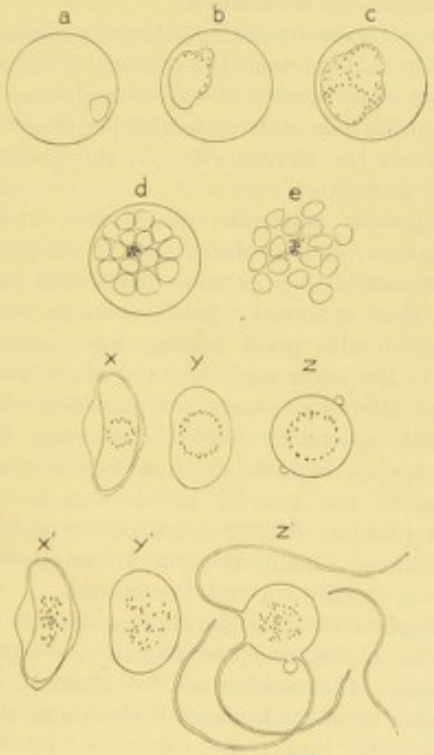


FIG. 29.

a—*z*, Phases in the asexual development of the malignant malarial parasite; *x'*—*z'*, phases in the sexual development.

and rounded, not tapering to sharp points as a true crescent. These bodies always contain pigment, and this pigment is never in a solid block, but is always

composed of discrete particles grouped in a cluster near the centre of the parasite.

These gamete forms are best observed in fresh fluid blood films, and it is only in such films that the subsequent changes can be followed.

As it is not easy to distinguish the gamete of tertian and quartan malaria from the sporocyte, the subsequent changes are most readily observed in the case of malignant or sub-tertian malaria, as the peculiar "crescent" shape renders the identification of the gamete easy in this class of malaria.

These changes only take place when alterations in the blood occur, such as abstraction or addition of water. The crescents lose their peculiar shape and become first oval and then spherical. Small portions, one or two, are extruded, the polar bodies, and remain usually adherent to the outer surface of the altered crescent.

Of these altered crescents, a proportion which varies in different specimens throw out long, filamentous flagella, varying in number from two or three to six. These flagella are actively motile and lash about in the blood plasma or over neighbouring red corpuscles for some time. Finally they break away and can be seen moving rapidly through the plasma.

The crescents which undergo this change and flagellate are the males, and the flagella are equivalent to spermatozoa. The residue of the crescent is a small protoplasmic mass containing all the pigment. It soon dies and is either broken up or devoured by a leucocyte.

The other altered crescents do not flagellate. After the extrusion of the polar bodies they retain their spherical shape, but the pigment in the interior is often in a state of violent agitation.

Very rarely a flagellum that has broken off the male crescent is seen to enter this rounded body, and is absorbed by it. These mature crescents which do not

flagellate are the females, and the entrance of the flagellum fertilises this female gamete. After fertilisation further changes take place, the pigment becomes violently agitated and the whole body changes shape, one end becomes conical, and the body becomes actively motile, moving steadily through the blood serum.

This fertilised female is known as the "travelling vermicule," and passes into the outer wall of the mosquito's stomach, where it becomes encysted and forms the *zygote*.

The male and female crescents can often be distinguished in the freshly-shed blood by the arrangement of the pigment. In the female there is usually a clear space in the middle surrounded by pigment, whilst in the male no such clear space is present and the pigment is less in a ring and more in a clump than in the female.

The young forms of crescents are sometimes found in the brain, spleen and elsewhere; they can be distinguished by their shape and the tendency of the pigment to be arranged in a central, irregular clump, and not in one mass.

The same series of changes occur in the gametes of benign tertian and quartan malaria after the blood is shed.

The gametes stain rather feebly with basic stains. The outlines of the red corpuscles which contain them can usually be made out, though, as all the hæmoglobin is absorbed, the remnant does not stain, or only faintly, with eosine.

The gametes contain chromatin in considerable quantity, but this chromatin, though it stains with the red of the polychrome methylene blue, does not stain with hæmatoxylin or most basic stains.

Stained by Leishman's method, the gametes of benign tertian and quartan are easily recognised, as the chromatin granules are collected in a clump surrounded by an unstained area free from pigment (Plate IV., 6).

In the crescents the chromatin in the female is collected into a solid block in the centre, and round this the pigment is arranged. In the male there is no central block of chromatin, but numerous scattered particles mixed up with the pigment. In the males the chromatin may be very abundant (Plate IV., 18, 19).

To make permanent preparations showing the changes that occur in shed blood, it is necessary to prevent the blood from drying and examine at intervals of a few minutes. For this purpose the slides with the fluid film on them must be kept in a moist chamber. This is easily done by cutting windows in a folded piece of blotting paper and placing this blotting paper on a slab. The blotting paper should be moistened, and the windows each covered with a slide on which is the wet film face downwards. The evaporation from the damp blotting paper will render the air so moist that evaporation from the film will be very slow.

One of these slides can be taken off and allowed to dry every five minutes, and in this way we have a series of blood films five, ten, fifteen, twenty minutes, or more, after the blood has been shed.

To obtain stained specimens of flagellating bodies thick films can be used, and when allowed to dry the hæmoglobin may be removed by placing the slide in water. After this the film is again allowed to dry, fixed in alcohol, and stained with a strong basic stain, such as carbol fuchsin.

To observe the changes that occur in the arrangement of the chromatin these decolourised films cannot be used, but the specimen with a moderately thick film must, after drying, be stained by Leishman's method. In both male and female a portion of the chromatin will be seen to be extruded in the polar body. The remainder increases in amount, and most of it in the male will be seen in the form of nodules at the periphery

of the altered crescent. Finger-like processes of the protoplasm will be seen to project from the vicinity of these masses, at first without any chromatin, and these processes elongate and form long, slender flagella without chromatin. Ultimately, however, the chromatin enters the flagella as a long, thin filament, leaving a mere residuum in the remnant of the crescent at the base of the flagellum. When the flagellum breaks loose it has this chromatin filament running nearly its whole length. Even when all the flagella have broken away there are still remnants of the chromatin in the protoplasmic residual mass left behind.

In the female the chromatin forms a less compact mass after the extrusion of the polar bodies, and it is with this mass that the chromatin of the flagellum which fertilises it probably fuses.

Minor differences in the crescents as regards shape, staining, reaction, and colour of the pigment are described by those who subdivide the malignant or sub-tertian into three species.

As regards the genesis of the gametes, suggestions have been made from time to time that they may be formed by the union of two young parasites in one corpuscle. Two, or even three or four parasites are not uncommonly found in one red corpuscle. These parasites may be in actual contact with each other, but there is no satisfactory evidence that conjugation or fusion of two such parasites ever take place. On general grounds such a method of forming the male and female sexual forms has nothing to support it.

The difference between the three main species of parasites are shown in tabular form on the next page.

Mistakes can be made with every method of blood examination, but most of them after a little experience are easily avoided.

In fresh fluid blood films the following are often mistaken for non-pigmented parasites :—

	[1] Length of Cycle	[2] Number of Spores	[3] Activity of Movement	[4] Selective Sites for Sporulation	[5] Character of Pigment	[6] Effect on Red Corpuscle	[7] Form of Gamete
TERTIAN.' (BENIGN TERTIAN).	48 hours.	15 to 25.	Very active.	In the circulating blood; common, but most abundant in spleen.	Yellowish-brown. Fine granules.	The corpuscle becomes swollen and pale. With special stains Schüffner's dots are found.	Rounded body.
QUARTAN. (BENIGN QUARTAN).	72 hours.	8 to 12.	Usually sluggish.	In circulating blood.	Black coarse granules.	The corpuscle becomes smaller and darker.	Rounded body.
MALIGNANT TERTIAN. (SUB-TERTIAN). AUTUMNO-ÆSTIVAL.	Variable and difficult to determine; probably about 34 to 48 hours.	Varies greatly in some cases; 7 or 8 are the common numbers whilst in other cases 20 or more spores are common.	Very active.	In internal organs, brain, lungs, intestines, &c. Very rarely found in circulating blood.	At first fine and black, but aggregate into masses earlier than in the other parasites.	At first little change, but later corpuscle is decolourised.	"Crescent," or sausage-shaped body.

(1) The normal lighter colour of the central portion of the corpuscle, due to the bi-concave shape of the red corpuscle. The gradual shading and the absence of any definite edge to the lighter part is usually sufficient to prevent this error, and familiarity with this appearance in normal blood is of importance.

(2) Vacuoles or tears in a blood corpuscle are distinguished by the very sharp, abrupt edge of such a vacuole, and by the oscillatory motion of the edge. It can be generally seen that whilst in a parasite there is a faint opalescence, in the vacuole the space is perfectly clear (fig. 30*c*).

(3) Blood plates resting on a blood corpuscle are in some cases difficult to distinguish. Round such blood plates there is usually a ring where the hæmoglobin has been pressed out of the corpuscle, and in some cases by focussing we can determine that the body is one which is on and not a part of the red corpuscle.

(4) Small particles resting on a corpuscle will displace the hæmoglobin beneath them and cause a lighter coloured patch in the corpuscle. Such particles, if dark, are often mistaken for pigment, and the pale area is taken for the parasite.

(5) Crenations, particularly when they occur as projections on the upper or lower surface of a corpuscle, are frequent sources of error. The effect of focussing, or alteration of the illumination, will show the true nature of these crenations (fig. 30, *a* and *b*).

(6) Bent or twisted "buckled" corpuscles may cause confusion (fig. 30, *d* and *e*).

Many effects are mistaken for pigmented parasites. Some of these are due to insufficient illumination, as refraction effects with a dim light closely simulate grains of pigment. Crenated corpuscles, leucocytes, &c., are thus sometimes taken for pigmented parasites. Full illumination will dispel this illusion. Particles of dirt,

or epithelial fragments with specks of dirt adhering, usually overlap at one edge or other a red corpuscle on which they lie. If they do not, by focussing it can often be determined that they lie on or beneath the red corpuscle. In most cases such fragments can be distinguished by their sharp angular outline, the irregularity in the size of the grains of dirt they contain, and by their high refractive index.

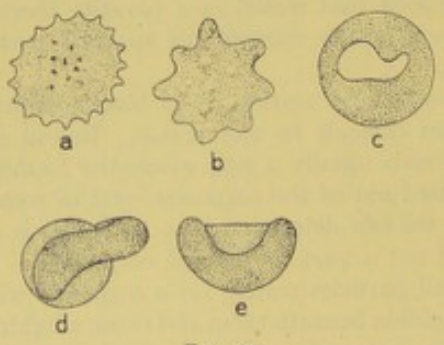


FIG. 30.

a, *b*, Crenated corpuscles; *c*, vacuolated corpuscle; *d*, *e*, buckled corpuscles.

Flaws, specks of dirt, or grease on slides or cover-glasses may cause confusion and be distinguished in the same manner. In any case of doubt it is well to touch the edge of the cover-glass with a needle whilst observing the object, and in that way it will be seen that the movement of the object is independent of the corpuscle that was supposed to contain it.

In stained specimens there are similar fallacies, and in addition, dirt from the stain, precipitated grains of stain, yeast cells, or other micro-organisms, may be present. It is well in any case of doubt to examine some part of the slide where the stain has extended

beyond the blood film, and see if the same appearances are presented there.

In the great majority of cases, if the appearances met with in normal blood have been carefully studied, particularly the blood plates and the various forms of degeneration of blood cells and of stained precipitates, mistakes are rare. Very rarely do we get an appearance from stain precipitates deposited on a red corpuscle that is difficult to distinguish, and therefore we should avoid diagnosing malaria from a single body believed to be a parasite. It is better in case of doubt to look carefully for a second parasite.

Crescents should never be diagnosed on the ground of the shape only. A crescent always contains pigment, and is longer than the diameter of a red blood corpuscle, and stains with basic stains. Three blood plates arranged in a row may be about the same size and shape as a crescent, but do not contain pigment or stain like a crescent. A transformed or altered crescent can be mistaken for a quartan parasite.

Groups of blood plates are sometimes taken for sporulating bodies, and if they surround, as they may, a mass of dirt, the mistake is easily made. Even in fresh fluid blood the peculiar appearance of the edges of blood plates should prevent this mistake, and in stained specimens the manner in which the blood plates stain will enable them to be recognised.

Imperfect fixation is a cause of some errors. In specimens fixed by heat, or fixed in alcohol that has absorbed water, small round bodies, artificial vacuoles, water or air, are often found in the red cells. They may be numerous in each corpuscle, or only one or two may be present. The sharp edge and high refractility of these bodies, as well as the variation in size, distinguishes them from parasites.

Familiarity with the appearances of blood prepared

in different ways is necessary, so that these appearances will cause no difficulty, and the recognition with certainty and rapidity of parasites will then be easy.

Hæmosporidia are found in other animals. The African monkeys are said to harbour a parasite closely resembling the human malignant tertian. Bats harbour parasites not unlike the human quartan, but these are not the same as the human parasites, which cannot be transmitted to these animals. No warm-blooded animal but man has been found to harbour the human malaria parasite. Cattle and horses may be infected by hæmosporidia which do not form pigment (Plate II., 13 to 16). The number of spores formed is usually two only, though four are occasionally found in the horse. Similar parasites have been found in dogs.

Birds harbour two species of hæmosporidia—*halteridium* and *proteosoma*. The most common is the halteridium; it is found in many species of birds—pigeons, crows, jays, finches, parrots, kingfishers, &c., and often the great majority of the birds of a susceptible species are infected (Plate I., 24, 25; III., 14, 15).

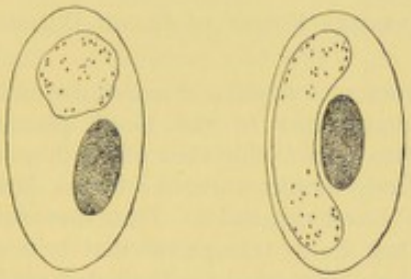
The mode of transmission is unknown. With this parasite MacCullum first demonstrated the conjugation of the hæmosporidia.

The other species of this class of parasite—*proteosoma*—is found in comparatively few species of birds. Amongst these the Indian sparrow is one of the most important. It was with this parasite that Major Ross first demonstrated completely the sexual cycle in a mosquito.

These avian parasites both form pigment: they can be distinguished from each other by the mode of growth, as the *proteosoma* displaces the nucleus of the red corpuscle (fig. 31, *a*), whilst the *halteridium* grows in an elongated form beside the nucleus (fig. 31, *b*).

In blood of amphibia the parasites found are *hæma-*

gregarina. These parasites, of which the drepanidia are the best known, at first are intracorpuscular, but after a time break out of the red corpuscle and are found freely swimming in the plasma. They are elongated bodies, have no vesicular nucleus, do not form



a *b*

FIG. 31.

pigment, and the chromatin is arranged towards the centres as a radiating mass. Their method of reproduction is unknown. Several species have been described which differ in size, shape and effect on the red corpuscle containing them and in the manner in which they stain (Plate III., 16 to 21).

CHAPTER V.

Parasites Found in Blood Plasma.

Mastigophora, or flagellated unicellular animal organisms, are represented in the blood plasma by the trypanosomata. In freshly-shed blood they are readily seen as actively motile, worm-like bodies darting about between the blood corpuscles. Their movements are so rapid and they are so transparent that it is difficult to make out their form clearly in the living condition.

The largest trypanosomata are found in fish (Plate I., 26), both fresh and salt water. They can readily be demonstrated in small fish by cutting off their heads and making a smear on the slide with the cut surface. These smears can be examined fresh by placing a cover-glass on the top of the exuded fluid, or the films can be dried and stained.

Trypanosomes are found in some birds. The method of examination of the blood for these is the same as that required for the hæmosporidia. If a small bird is to be examined it is held in the palm of the left hand and one leg is allowed to protrude between the fingers. A needle is then inserted deeply into the vascular pad surrounding the root of the claw and left there for half a minute. On squeezing the leg so as to force the blood towards the claw the blood will exude in drops, and films can be made as with human blood. With larger birds an assistant is necessary to hold the bird, and the bird should be wrapped in a thick cloth for the protection of the assistant.

The trypanosomata that have attracted most attention are those of the mammalia. Several species are known; they closely resemble each other in their appearance, but differ in size and shape to some extent; also in the positions they assume and the way in which they stain (Plate I., 26, 27, and Plate IV., 21, 22). The only certain method of differentiation is by inoculating with the blood a series of animals, and it will then be found which animals are immune and which are susceptible. Some species are pathogenic and others not. For such inoculations the blood must be mixed with some fluid that will prevent coagulation. Sodium citrate solution 10 per cent. may be used, and the blood should be diluted with one-twelfth of this solution. Others use a weaker solution of citrate of soda, 1 per cent., and dilute the blood more freely. Injection of such diluted blood into the subcutaneous tissues will lead to infection with trypanosomata of the animal which has been injected.

The more important of the trypanosomata are :—

(1) Those found in a large proportion of the rats in both tropical and temperate climates. These are non-pathogenic to rats, and all other animals experimented on are insusceptible to the infection (*T. Lewisi*, Plate IV., 21).

(2) Nagana or "Tsetse Fly Disease." The trypanosoma of this disease (*T. Brucei*) can be inoculated into a large number of wild and domesticated animals, but man is insusceptible. To cattle, horses, donkeys, dogs, rats, &c., this parasite is pathogenic, but the time required to cause death varies greatly in these animals. Wild game, and particularly the buffalo, harbour the parasite, which appears to be harmless to them (Plate I., 27).

(3) Surra (*T. Evansi*). A disease fatal to horses; cattle usually recover. It occurs in India, Philippines, &c.

(4) Dourine (*T. Equiperdum*). Europe. Rats, dogs, rabbits, &c., are susceptible. Cattle, sheep and goats are refractory.

(5) Mal de Caderas. South America. Cattle are immune; most of the other animals are susceptible.

(6) Trypanosoma Theileri. South Africa. Cattle only are susceptible. No other animal has yet been inoculated successfully.

(7) Trypanosoma Hominis v. Gambiense* (Plate IV., 22). Man is insusceptible to all the above-mentioned trypanosomata, but on the West Coast of Africa another species has been found in man. In the cases observed the parasites have been in small numbers, though the symptoms, constant irregular pyrexia, erythematous rash, and enlargement of spleen and liver, have been marked. It is stated that white rats and monkeys are susceptible.

For the examination of blood for trypanosomes films prepared as for malaria are the best, as the parasite will then be seen undistorted. When the parasites are scanty and for purely diagnostic purposes, thicker films decolourised by the action of water may be used. In some infections the parasites can only be found by injecting a highly susceptible animal with the blood of a suspected case, as a large infection may then result in the animal which has been injected. This proceeding is necessary in many cases of dourine. No susceptible animal is yet known for the human trypanosome, and therefore for this parasite tedious and prolonged microscopic examination of blood films are necessary. In centrifugalised blood the parasites accumulate in the upper part of the mass

* Recently a trypanosome has been found by Castellani in the cerebrospinal fluid and blood of cases of sleeping sickness in Uganda. This trypanosome, which has received the name of *Trypanosoma Castellanii* by Kruse, may possibly represent a new species.

of red corpuscles and can be found there more readily than by the ordinary method.

Trypanosomes stain rather feebly with most basic stains, hæmatoxylin, methylene blue, &c. A stronger basic stain, such as carbol fuchsin, should therefore be used. Clearer specimens are obtained by diluting the

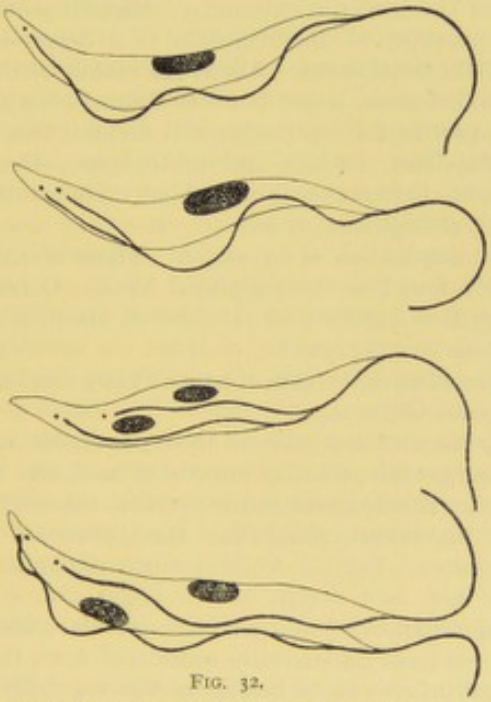


Fig. 32.

stain with two parts of water and leaving to stain for ten minutes.

Good results can also be obtained by overstaining with this stain and then decolourising with $\frac{1}{2}$ per cent. solution of glacial acetic acid in water, but the parasite is often swollen and distorted, though quite recognisable.

Leishman's stain, used as for other blood work, gives

excellent results with fresh specimens and shows well the various points in the structure that have been determined. The body is elongated and the posterior extremity is bluntly truncated, whilst the anterior is prolonged into a long flagellum, rarely into two. The flagellum is continued as a definite curved rod in the body of the parasite nearly to the posterior extremity. Slightly posterior to the termination of the flagellum is a deeply-staining nodule—the centrosome. About the middle of the body is a rounded mass, larger but less defined—the nucleus. In fission forms the centrosome first divides, then successively flagellum, nucleus and protoplasm. The protoplasm with Leishman's stain is blue. The centrosome, nucleus and flagellum are red.

The multiplication is by fission. These fission forms are rarely found in the peripheral blood. Occasionally there are two flagella with no signs of fission in centrosome or nucleus (fig. 32).

Transmission in the case of nagana is by flies belonging to the genus *Glossina*.

The transmission is believed to be direct, the trypanosomes being taken from an infected animal, and without any further development in the fly enter the next animal bitten. No sexual phase has been observed in the trypanosomes. Further work is much required on this subject.

The spirillum of relapsing fever—*Spirillum Obermeyeri* (fig. 33)—is most conveniently considered here, though it is generally believed to belong to the vegetable micro-organisms. They can be seen in fluid blood films made as for malarial blood. The organisms are very transparent and can only be seen with the diaphragm nearly closed in fresh fluid preparations. They are then seen as fine, transparent, thread-like bodies, which are in active movement and coil and uncoil themselves. They are rarely seen in the corkscrew-like forms which are commonly drawn as representing them.

Dried films must be thin. The spirilla stain with all basic stains, but not intensely, and are best demonstrated by the use of the stronger basic stains, such as carbol fuchsin (Plate IV., fig. 23).

In old films stained by Leishman's method the spirilla stain blue and do not show chromatin. With old films this is not conclusive.

The disease can be reproduced in monkeys.

There is leucocytosis and marked relative increase of the polymorphonuclear leucocytes. This increase persists to some extent in the periods of apyrexia, so that a differential count of the leucocytes may exclude malaria.

The spirillum never shows any signs of division in the blood, and in human blood has no tendency to great variation in length. It is found in the plasma, never in the blood corpuscles. The spleen enlarges, and in fatal cases spirilla are found in large numbers in that organ. The organisms are found in greatest number during the first pyrexial period. In the apyrexial period they are not to be found, and in the subsequent pyrexial attacks they are found in much smaller numbers than in the primary attack. In cases where the disease passes on into a chronic condition of irregular pyrexia—secondary fever—it is exceptional to find the parasites during that period (fig. 33).

The spirilla found in the mouth and sometimes in fæces more closely resemble the recognised bacterial spirilla.

The other animal parasites found in human blood belong to the higher orders of animal life. One, the *Bilharzia* (*Schistosoma hæmatobium*), frequents the veins of the portal system.

The males are infolded in their entire length, and thus form a deep grove or incomplete tube—the gynæcophoric canal—in which the thinner female is contained.

They are flattened, worm-like bodies, and are bisexual.

The males are the larger, 12-15 mm. in length and 5 mm. in breadth. At the posterior termination of this canal is the sexual opening. There is no penis. The female is longer than the male, 16-20 mm., but thinner, 2 mm. in breadth, and therefore protrudes from each end of the gynæcophoric canal. The sexual orifice is close to the

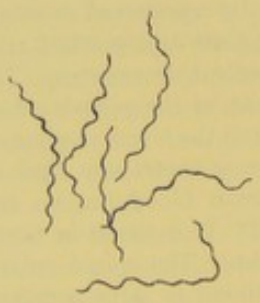


FIG. 33.—SPIRILLA.

ventral sucker. There are two suckers on the front of the body, an oral and ventral, and the intestinal canal, which frequently contains blood, begins and terminates at these orifices respectively. The eggs, which are provided with a sharp spine, do not pass with the blood stream but towards the pelvis, where they become extra-

vascular and pass through the mucosa of bladder or rectum, or in some cases higher up in the intestine or urinary tract, into the urine or fæces.

Their further development will be considered with those excretions.

NEMATODES.—One species of filaria in the adult form has been once found in the circulatory system of man by Magalhães in Brazil, but no further observations have been made. The worms were found in a blood clot in the left side of the heart.

In the lower animals nematode worms are not uncommon in the blood.

Filaria immitis is found in the right side of the heart and pulmonary vessels of the dog, and in the East and in some of the Pacific Islands it is exceptional to find a dog free from these parasites. When the worms are in large numbers cardiac dilatation and death results.

The various nematode worms in horses and other animals cause verminous aneurisms.

Filaria sanguinis hominis. Of the human filaria the adults are found in several tissues in the body, whilst the embryos may be discharged through an aperture in the skin, as in guinea-worms, and probably in *Filaria volvulus*, a worm found in a subcutaneous cyst in a patient in Sierra Leone. In the ones we are at present more specially interested in they find their way into the blood and circulate with that fluid.

The filarial embryos as seen in fresh blood are clear, transparent, worm-like bodies, which are in active movement. They are most readily found in a fresh fluid blood film, as the active movements and the disturbance in the red corpuscles set up by their movement catch the eye. An inch or two-thirds inch objective is quite sufficient magnification for the detection of the commonest filariæ, but it is better to use a half-inch, as the smaller species may be overlooked with the two-thirds

objective. The film must not be so thin as that used for examination for malarial parasites. No special precautions are required, and sufficient blood should be taken to completely fill the space between the slide and cover-glass. As the slide must be kept for a sufficient period to enable the movements of the worm to cease, the cover-glass should be ringed with vaseline to prevent evaporation of the blood.

To examine the embryos in detail higher powers, including an one-twelfth oil immersion, are required. At first the movements of the filaria are so active that it is impossible to examine it with these objectives, but after some hours the movements become much more sluggish, and finally cease. The best time for examination is just before the cessation of movement and the death of the embryo. The points to observe in the examination of the fresh embryos are :—

(1) The character of the movement and whether active locomotion takes place or whether the movement, however active, leads to no progression.

(2) The size of the embryo. This is of the greatest importance, as measurements of dried specimens vary greatly with the rapidity with which the film has dried.

(3) The shape of the embryo and that of the two ends.

(4) The presence or absence of a loose sheath.

(5) Any details of structure, and particularly the presence, position and character of any contractile vesicles, the so-called V spots, and the cephalic movements and any appearance of armature require close examination.

Embryos can also be observed readily in dried films.

The blood films for diagnostic purposes should be as thick as possible. A convenient way of making them is to allow three or four large drops of blood to fall on a

slide close together and smear them together into a square about two-thirds of an inch in diameter (fig. 34). Allow to dry, protecting the films from insects during the process. Such a film will be so thick as to be almost opaque. It must not be fixed. When quite dry place in distilled water and leave in the water till the hæmoglobin is all dissolved out. It is best to have the film side downwards in the water but not resting on the bottom of the vessel. As the hæmoglobin dissolves out it will fall to the bottom of the vessel. It will be found

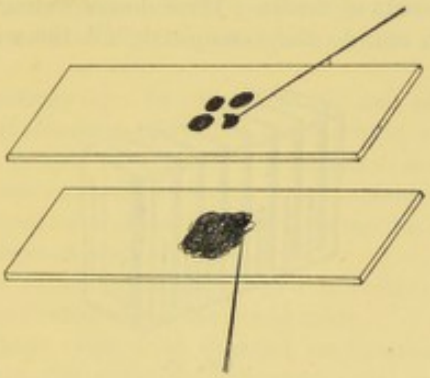


Fig. 34.

better after a few minutes to transfer the slide to clean water, so that it is easy to observe when the hæmoglobin is all removed.

Remove the slide from the water and examine at once whilst still wet. The white corpuscles will stand out from the film as refractile spots and the white colourless worms will also stand out brilliantly.

If it is preferred to stain the specimen, any basic stain gives good results. Weak carbol fuchsin is perhaps the best of the aniline stains. Hæmatoxylin gives good and

permanent results, but the sheathed filariæ do not stain rapidly. If the hæmatein mixture is used, fifteen or twenty minutes will be required and the slide should then be left in water for ten minutes.

A good many slides can be stained together. For this the staining vessel (fig. 35) is convenient.

Counter-staining brings out nothing more, but eosine may be used for this purpose.

The shape of the worm is well shown in a specimen stained with hæmatoxylin, and also the sheath if present. The body of the worm is found to contain a core of deeply-staining points or nuclei. These do not extend to either extremity, nor do they completely fill the worm, as a

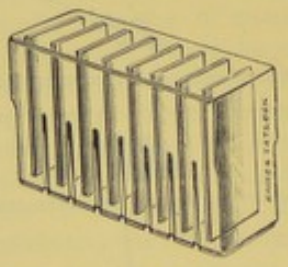


FIG. 35.

clear, unstained portion is left on each side. This unstained portion must not be mistaken for the sheath. The sheath will be faintly stained and only clearly seen at the two ends, where it will be found flattened on itself and often folded sharply like a piece of ribbon.

In the nuclear core complete or incomplete gaps in the mass of nuclei will be seen in most filariæ. For each species the position of these gaps is constant, or nearly so, and consequently the exact position of these gaps is important for the differentiation and identification of species from the examination of these embryos (Plate II., 17, 18, 19).

Embryos of some species of filaria are not found in the same number all through the twenty-four hours. During a part of this period they may be found in numbers, whilst a few hours later they are found with difficulty or not at all. Thus one species has a periodicity which is called *nocturnal*, because the embryos are found in largest numbers in the peripheral blood at night; in other species embryos are only found in the day time, and are said to have a diurnal periodicity. Embryos of other species are found in fairly equal numbers at all times of the day and night.

In any investigation of the periodicity of filarial embryos it is essential that the blood examined should be measured.

The periodicity can be altered in the case of *Filaria nocturna* by changing the habits of the host, and cases are fairly common in which the periodicity is reversed without known cause. It is still more common to find small numbers of *Filaria nocturna* during the day and larger numbers at night.

The chief points of difference in the various embryo filariæ are indicated in the subjoined table.

These points require no detailed explanation. It is well to draw the embryos accurately with a drawing camera or camera lucida.

By substituting a scale for the object a scale can also be drawn on the same paper and measurements made from this, which are easier and usually more accurate than measurements made with a micrometer eye-piece.

Periodicity refers to the appearance of embryos in the peripheral blood.

With regard to this periodicity, it was not definitely known what became of the embryos during the time they were absent from the peripheral blood. *Post-mortem* examinations, however, have shown that in the case of persons harbouring this filaria who die during the day,

the embryos are found in greatest numbers in the lungs and large vessels, though some may be found in the vessels in other viscera.

Name of Embryo	Length	Greatest Thickness	Sheath	Shape of Head	Shape of Tail	Periodicity	Distance of Head Cap from Head	Adult (known or suspected)
Filaria nocturna	mm. ·317	mm. ·0075	Present	—	Sharply pointed	Nocturnal in peripheral blood	mm. ·052	*F. Bancrofti.*
Filaria diurna	·317	·007	Present	—	Sharply pointed	Diurnal in peripheral blood	—	*F. Lea.*
Filaria perstans	·195	·0045	Absent	—	Blunt, truncated	None	·03	*F. perstans.*
Filaria Demarquaii	·21	·005	Absent	—	Sharply pointed	None	·03	*F. Demarquaii.*
Filaria Ozzardi	·21	·005	Absent	—	Sharply pointed	None	·03	*F. Ozzardi.*
New Filaria *F. gigas* (Prout)	Decidedly longer and thicker than any of the above		Absent	—	Blunt	?	—	?

Sections of the organs of such a person show the filariæ in great numbers. The material may be imbedded in either celloidin or paraffin and should not be too thin, as, unless rather thick, such short lengths of the filaria are cut that they are not easy to recognise.

Hæmatoxylin solution, two minutes, is quite sufficient to stain the embryos, and there is no need to counter-stain. Transverse and oblique sections of numerous

embryos will be found. In places longer lengths, or even complete embryos, which were lying in the plane of the section, may be seen.

As far as is known, no further change takes place in the human filarial embryos in the blood or human tissues, but there is evidence that some degree of growth whilst circulating in the blood does take place in some of the avian filariæ.

Of the human filaria, the next stage of growth occurs in several species of mosquitoes of different genera—*Culex*, *Anopheles*, *Panoplites*, *&c.*—and when a certain stage of maturity is reached the embryos are injected by the mosquito into man. At this stage the embryos are 1·5 mm. in length, and the differentiation of sexes is not complete.

The further development in man has not been traced, but the adult forms of the species, *Filaria nocturna* (*Filaria Bancrofti*), have been found by many observers always in, or in connection with, the lymphatic system. The other human adult filariæ, *Filaria perstans*, *Filaria Demarquaii*, *Filaria Ozzardi* and *Filaria loa* (probably the adult form of *Filaria diurna*), are found in connective tissue, either subcutaneous or in the subperitoneal tissues.

The adult human filariæ are not very readily found. *Filariæ Bancrofti* are found in lymphatics in almost any part of the body, but as a rule, in the cases of elephantiasis, the adults are long dead and only the positions they once occupied indicated by lymphatic obstruction.

Filariæ perstans, though smaller, are more readily found, as they occur at any rate in greatest numbers in subperitoneal connective tissue, particularly at the base of the mesentery.

Filaria Demarquaii has been found by Dr. Galgey in the same position, and *Filaria Ozzardi* has been once found in the subserous connective tissue of the anterior abdominal wall.

Filaria immitis, the "worm in the heart" of dogs, is found in the cavity of the right side of the heart and the pulmonary vessels. When only one or two worms are present they are usually in the smaller pulmonary arteries.

Avian filariæ occur in many positions. Some species are found in loose connective tissues, as in the neck, others in the limbs, and particularly in thickenings about the claws; others in the submucous tissues, as in the crop; and others in the blood-vessels, and even in the pouches formed by the semi-lunar valves. Adult filariæ are easily mistaken for empty blood-vessels, small nerves and shreds of fibrous tissue. They are more readily recognised with slight magnification, and for this purpose, as well as for dissections of insects, &c., a watchmaker's glass of about 5″ focal length is very useful. The horn ones are best, and should be perforated at the sides, as otherwise moisture condenses on the lens. The advantage of these glasses is that both hands are free, and it is easy to learn the use of this simple lens.

In searching tissues for filaria a dark surface, such as a slab of slate, makes a good background, and the rough surface of the slate prevents the specimen slipping about. The tissue should be kept wet with normal saline solution, as this keeps it transparent.

The dissection should not be made with the tissues *floating* in water or salt solution, as strands of tissue are much more readily twisted or ravelled out if floating. The tissues must be kept wet.

Some authors construct a formula for the description of filariæ based on the relative positions of various structures and the measurement of the worm at these places. The unit of measurement is the one-hundredth part of the length of the worm, so that the measurements are percentages of the length. Five measurements are taken by the author—Cobb—commencing from the head: the base of the œsophagus, the nerve ring, the cardiac con-

striction, the fourth at the vulva in the female and the middle of the male, and the fifth at the anus (fig. 36).

Many of these points are very difficult to make out in the human filariæ. The first of them in life is very variable in the same individual. As the head and neck are capable of considerable contraction, the head cannot be taken as a fixed point to serve as the basis of a series of measurements. The whole formula also is based on the assumption that the proportions of various parts of the body are constant in different individuals, which, according to Shipley, is not certain.

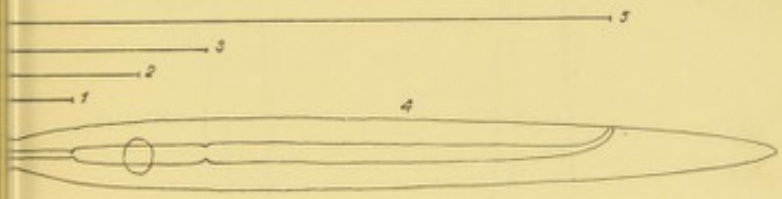


FIG. 36.

Though I do not consider that this graphic method is applicable in many cases, still, where possible, it may be given.

The human filariæ resemble each other rather closely in their adult forms, and some of them require very careful examination for differentiation. Measurements of the head and tail, making due allowance for the contractility of the worms, are of great importance. Particular attention must be paid to the transparent cuticle, as there are important differences in its arrangement in different species, and these differences are constant for the individuals of each species.

The measurements should be made, where possible, on the fresh worms, as serious shrinking and distortion occurs with most reagents. Alcohol and spirit cause great

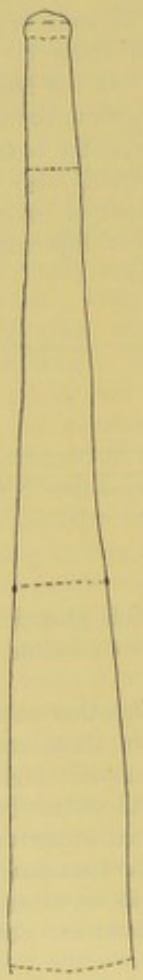


FIG. 37.
Head of *Filaria Bancrofti*, ♀.

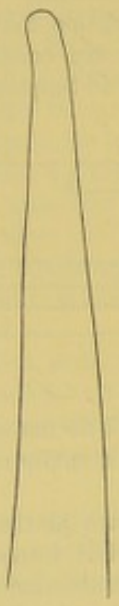


FIG. 38.
Head of *Filaria Ozzardii*, ♀.

distortion. This can be diminished by placing the specimen first in dilute spirit, 1 to 3 of water, for a few hours, and then gradually increasing the strength, but however carefully this is done the distortion is great. Much less distortion is caused by spirit if the specimen is first hardened in formalin 2 per cent.

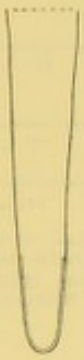


FIG. 39.
Tail of *Filaria Bancrofti*, ♀.

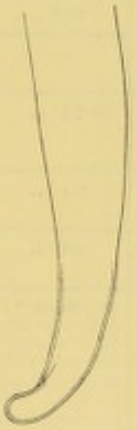


FIG. 40.
Tail of *Filaria Ozzardi*, ♀.

Glycerine at first causes swelling, though when left long in the glycerine there is a return to a more natural condition. The specimens so prepared are much softened and can very readily be flattened out, and whilst thus gently compressed between two slides, be hardened in methylated spirit and finally in alcohol, and mounted after clearing in oil of cloves. Such specimens are very transparent and do not show much detail; if, however, they are slowly stained with very dilute solutions of stains, such as dilute borax carmine, before placing in glycerine, many details of structure are brought out well. They can also be stained with well-diluted hæmatoxylin

	F. Bancrofti		*F. Perstans*		*F.*
	Female	Male	Female	Male	Female
Length	80-90 mm.	44 mm.	70-80 mm.	45 mm.	81 mm.
Greatest breadth ...	·23 ,,	·1 ,,	·12 ,,	·06 ,,	·21 ,,
Diameter of head ...	·055 ,,	·05 ,,	·07 ,,	·04 ,,	·05 ,,
Character of cephalic end	—	—	—	—	—
Distance of genital pore from head (female)	·66-·75 ,,	—	·6 ,,	—	·71 ,,
Diameter at point of genital pore ...	·14 ,,	—	·07 ,,	—	·12 ,,
Distance from tail of anus	·225 ,,	—	·145 ,,	—	·23 ,,
Cuticular thickening on tip of tail ...	None.	None.	Double terminal cuticular thickening.	—	None.
Spicules (male) ...	—	Two unequal, anterior and posterior, both retractile.	—	Two unequal spicules.	—
Papillæ (caudal) ...	None.	None.	None.	Four preanal and one postanal. Very close to opening of cloaca.	None.
Habitat	Lymphatic system.		Connective tissue.		
Geographical distribution	In most Tropical regions.		Africa (West Coast and Central), British Guiana.		Britis

	F. Demarquayi		*F. Loa*		*F. Megalhaesi*	
	Female	Male	Female	Male	Female	Male
	65-80 mm.	Not known.	50-55 mm.	30-35 mm.	155 mm.	83 mm.
	·21-·25 ,,	—	·55 ,,	—	·6-·7 ,,	·3-·4 ,,
	·1-·09 ,,	—	—	—	·06 ,,	·04 ,,
	—	—	—	—	—	—
	·76 ,,	—	2·35 ,,	—	2·56 ,,	—
	·1 ,,	—	—	—	·58 ,,	—
	·25 ,,	—	·3 ,,	1·75 ,,	·13 ,,	—
	Cuticular thickening over tip. Knobby and irregular in outline.	—	No thickening over tip. Two lateral alæ. Cuticular bosses not found at tip.	Thickening over tip. The "bosses" so abundant over the cuticle in the body of the worm are not found at the tip.	None.	None.
	—	—	—	Two unequal, anterior and posterior.	—	Two spicules.
	—	—	—	Three preanal pairs and two postanal. The last are very small.	—	Four preanal and four postanal.
	y subperitoneal.		Connective tissues, subcutaneous, subconjunctival, or in the deeper parts of the limbs.		Left side of heart.	
	West Indies.		West Africa.		Brazil.	

and subsequently slightly decolourised with dilute acid spirit ½ per cent. to show *in situ* eggs and embryos.

Many filariæ show fairly well when mounted direct in glycerine jelly, but these, after a time, become distorted. If previously hardened by placing first in 1 per cent.

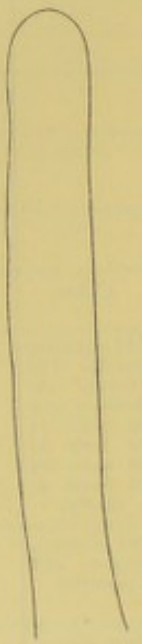


FIG. 41.
Head of *Filaria Demarquaii*, ♀.

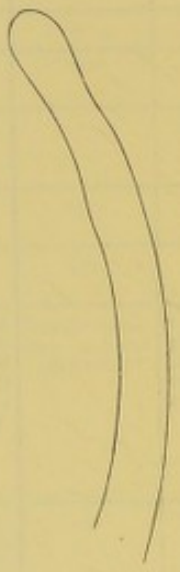


FIG. 42.
Head of *Filaria perstans*, ♀

formalin for two days and then in 2 per cent. formalin for two days, and then kept in 5 per cent. formalin for some days, they can be mounted in glycerine jelly, or even in Farrant's solution, and retain their natural size and appearance.

Some authors prefer to mount in a 2 per cent. formalin solution in a shallow cell, and ring with Hollis' glue, so as

to prevent evaporation, but such specimens in the long run nearly always dry up and thus valuable specimens are spoiled.

The different points enumerated in the tabular form are usually made out, but to see either the genital pore

FIG. 43.
Tail of *Filaria Demarquaii*, ♀.

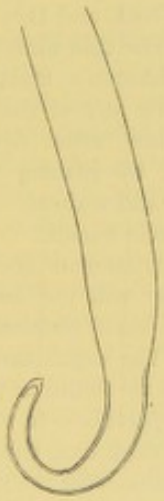


FIG. 44.
Tail of *Filaria persians*, ♀.

or the anal opening that portion of the worm must be viewed in profile. It is therefore necessary to turn the worm gently before mounting so that they can be seen. This can generally be effected by slightly moving the cover-glass by pressure of its edge with a needle so as to roll the worm over slightly.

The points of difference and resemblance are shown in the table (pp. 120, 121) for the known adult human filariæ.

EXAMINATION OF THE BLOOD FOR PATHOGENIC BACTERIA.—Most of the organisms found in blood films

are due to contamination with skin organisms during the preparation of the film. To avoid this the finger, which for this purpose is the most convenient part to examine, should be well washed with 2 per cent. lysol and then wrapped in a 1 in 500 sublimate compress covered with gutta-percha tissue for hours. The first drop of blood should be rejected as the most likely to be contaminated. Thick and thin films should be taken and rapidly dried. The thin films can be stained by Louis Jenner's and Leishman's stains, or the film can be fixed and stained by any of the methods used for bacteria. The thick films after drying can have the hæmoglobin removed by placing in sterilised water, and can then be fixed and stained. This method is particularly suited for tubercle bacilli.

Most organisms are present in such minute numbers that they will not be found by this method, and the drops of blood obtained should be used for making cultures. The organisms of plague, septicæmia, tubercle, &c., may be found in the blood.

Sarcosporidia.—In the muscles of some animals, such as pigs, rats, &c., tubular bodies are found. These are often visible with the naked eye. On microscopic examination these "tubes" are seen to be filled with refractile bodies, which are usually curved and sausage-shaped, but they may be round. The wall of the tube is in reality a capsule containing these sporidia. Nothing is known of the life-history or development of these bodies. Similar bodies have been found in man. They may also occur in the organs, for example the liver. The sarcosporidia stain readily with basic stains.

Little attention has been paid to these bodies. The separate organisms are invisible to the naked eye, but as they usually occur in masses and produce some colour change, appearing as light streaks or nodules, such streaks should be looked for and scrapings of them examined microscopically.

CHAPTER VI.

For certain purposes it is desirable that blood should be kept fluid and coagulation prevented. This is requisite when we wish to inject or transmit blood containing living parasites such as filariæ or trypanosomes.

If the blood be allowed to coagulate the parasites are usually entangled in the blood clot. To prevent this coagulation the blood should be quickly mixed with a citrate of sodium solution, a 10 per cent. solution should be used, and one part of this, if rapidly and thoroughly mixed, will prevent the coagulation of twenty-five parts of blood. Twice the amount of a 5 per cent. solution is somewhat easier to work with and equally effective, whilst others use a much larger proportion—physiological normal citrate of soda solution. In such mixtures the blood corpuscles are not destroyed.

Blood serum is required for several purposes, and particularly for the demonstration of the presence or absence of the specific agglutinins which cause the agglutination of the corresponding micro-organism.

The ingenious glass tubes devised by Wright are useful in obtaining and diluting such serum. The simplest form is to draw out the two ends of a piece of glass tube a quarter of an inch in diameter. In drawing out the tube it is well to rotate the tube in the flame until it is quite soft at the required place, and then remove from the flame and pull steadily and hard if the glass does not readily yield. In that way more uniform tubes are formed than if the traction be exercised whilst the tube

is still in the flame. The thin tube thus formed should be broken and at a convenient distance another portion of the tube should be heated and pulled out in the same manner (fig. 45). One of the capillary extremities should be sealed. A puncture with a broad needle or small knife should be made in the skin and the *upper* half of the unexpanded tube, that towards the sealed end, should

FIG. 44a.

FIG. 45.

be heated in the flame of a spirit lamp which is lighted and placed close at hand before the skin is punctured. Holding the lower part of the tube which has not been drawn out between the fingers to make sure that it is not too hot, the open drawn-out end is placed in the exuding blood. As the air in the tube cools and contracts the blood will be drawn up into the tube. If there is not enough or the blood is not drawn entirely up into the thick part of the tube by the time it is cool, the sealed end can be broken off and the upper end of the thick tubing again heated and the same end again sealed. The contraction of the air will be sufficient to draw more

NOTE.—We are indebted to the kindness of the Proprietors of the *Lancet* for the use of Fig. 44a.

blood up and the blood already in the tube higher up. When sufficient blood is in the tube and the tube is cool, the lower end through which the blood entered can be sealed. The tube is now placed on its side, horizontally, till the blood coagulates, and is then placed vertically, so that as the serum is expressed by the contraction of the clot it will run down into the narrow part of the tube. In this way clear serum, free from blood corpuscles, can be obtained without using a centrifuge. Capillary vaccination tubes can be used to collect the blood but will require to be centrifugalised to obtain clear serum.

Serum, however prepared, requires dilution for most purposes, and the degree of dilution is important. A very convenient method of obtaining any degree of dilution is by Wright's tubes. A piece of glass tubing is drawn out sharply in the middle so as to make a short, sharp constriction. About an inch and a half from this constriction on each side the tubing is drawn out into a long, thin capillary tube. One of these is broken off and sealed, and the other, preferably the more uniform and thicker, is touched with a file, broken off square and left open. The part above the middle constriction with the sealed capillary tube attached is called the air chamber, and that below in connection with the open capillary tube is called the mixing chamber. A narrow mark is made on the open capillary tube with a grease pencil about an inch or less from the open end. This distance depends on the calibre of the tube, the greater this is the shorter the distance from the open end to the mark, as the volume of the column of fluid between the open end and the mark is the unit of measurement. The finger or ear is pricked and from the blood obtained the serum is allowed to separate as above and blown out into a sterile watch glass. The air chamber is then well heated and the open end of the tube is placed in the blood serum till the serum

runs up to the mark. The tube is then removed from the serum and a little air enters the tube as the air in the hot air chamber contracts, the open end is then placed in the diluting fluid and withdrawn as soon as the fluid reaches the grease pencil mark; it is then withdrawn, but as soon as air has entered the tube it can be replaced in the fluid and again withdrawn when the mark is reached. This can be repeated as long as the air in the air chamber contracts. If repeated nine times there will be nine parts of the diluent to one of the blood serum. As the fluid by contraction of the air in the air chamber is all drawn up into the mixing chamber it can there be well mixed by rapidly rotating between the palms of the hands.

This procedure would give a dilution of one in ten, and by continuing the process greater dilutions would be obtained; but it is better, if high dilutions are required, such as 1 in 100, 1 in 1,000, after well mixing the serum and diluent, to expel a part of it into a sterilised watch glass by heating the air chamber.

The expelled diluted serum is further diluted in a second tube in exactly the same manner as the first dilution, and from this a third, and in turn a fourth dilution, can be made. The tubes with the diluted serum may be sealed up and kept for some time if necessary.

If it be desired, a known amount of a broth culture of an organism can be in the same manner drawn up into the mixing chamber and there mixed with diluted or undiluted serum.

Wright uses india-rubber teats to draw up the fluid, but as in the Tropics india-rubber does not keep well, the air chamber drawn out into a long capillary tube is more satisfactory. Wright, when using an air chamber, blows it out into a bulb so as to have a larger volume of air, but a smaller air chamber is sufficient in most cases, and if it cools too rapidly so that the air ceases to contract,

the sealed end may be broken off, and whilst the tube is still open the air chamber can be heated and the tube again quickly sealed. This can be repeated as often as one wishes if a large volume of serum or blood is required in the mixing chamber.

Instead of serum the blood itself can be mixed with a diluent in a similar manner, and the diluted blood used for counting leucocytes or red corpuscles. It is necessary that the diluent should be one that will prevent coagulation and will not cause destruction of the red corpuscles. Gower's solution is fairly satisfactory, or if it be desired to stain the leucocytes, Toisson's fluid may be used. The mixing for uniform and successful results must be done quickly, as otherwise part of the blood may coagulate or the corpuscles adhere together in masses.

In addition to agglutinins other substances may be formed in serum as a result of inoculations with organisms. These include the toxins and antitoxins, *i.e.*, the poisonous products of the growth of organisms or substances that are inimical to the growth of such organisms. Hæmolysins are formed as the result of the injection of certain organisms and other substances.

A class of substances which promise to be of much practical importance are the precipitins. It is found that if blood of one animal, as for instance man, be repeatedly injected into a rabbit, the constitutional disturbance set up by the injections becomes less and less, and after a few injections they cease to cause any disturbance. It is further found that the blood serum of this rabbit, immunised as to human blood, will give a precipitate when added to a solution of human serum or of closely-related animals, such as the ape, but not with solutions of serum of other animals, such as the rodentia.

Similarly, if a rabbit be immunised by repeated injections of the blood of any animal, horse, rat, pig, &c., the

serum of the rabbit will give a precipitate with solutions of the serum of the horse, rat, pig, respectively, or animals closely related to them.

This gives a new means of grouping animals and promises to be of practical and medico-legal value. The immunised or test sera can only be made where proper appliances are available, but appear to keep well.

The application of the test is easy. Clean white filter paper is soaked in the fresh uncoagulated blood of the animal and allowed to dry in the air. A portion of this paper soaked with the dried blood is treated with normal saline solution. The clear solution thus obtained is placed in a small test tube (Durham's tubes are suitable) and a few drops of the test serum are added. A precipitate indicates that the blood was either that of the animal against whose blood the rabbit was immunised or a closely-related one.

Dr. Nuttall has made observations on a large series of animals and the results obtained have been consistent, and in many ways have thrown light on the relationship of different animals. The filter papers soaked in blood and well dried in the air keep well and give the reaction after many months. It is important that blood should be obtained from any rare animal and examined in this way to aid in its classification. The filter paper or white blotting paper should be soaked in the blood and clots removed. The blood must be fresh. The paper should then be allowed to thoroughly dry and, if possible, wrapped in waxed paper and sent to England to be tested with the prepared sera.

Coagulation Time.—Blood varies greatly in the rapidity and firmness with which it coagulates, and the time required is influenced by various diseases. Methods of estimating the coagulation time for clinical purposes are not very satisfactory, and all determinations must be made at a constant temperature.

Wright's method is to draw up blood into a series of capillary tubes and attempt, by blowing at intervals of half a minute, to dislodge the blood. When it cannot be dislodged it has coagulated and the time it has taken is the coagulation time. This simple method appears to give as good results as any.

The specific gravity of the blood is another variable element and is not easily determined accurately with the small amounts of blood that can be used for clinical purposes.

Blood is dropped into a series of fluids of known specific gravity varying from 1035 to 1068, and the specific gravity of the fluid in which the blood neither sinks nor rises is of the same specific gravity as the blood. The fluids chiefly used are glycerine and water, or chloroform and benzol in varying proportions.

The reaction of the blood can be determined either by using glazed litmus paper previously soaked in chloride of sodium solution, or a plaster of Paris disc soaked in neutral litmus solutions.

In addition to hæmoglobin we may have in the blood in cases of jaundice bilirubin, and in some cases derivatives or modifications of hæmoglobin are present. A small direct vision spectroscope is the most satisfactory method of determining the presence of these substances. The blood should be laked by the addition of distilled water to render it sufficiently translucent. If it be desired to determine the presence or absence of hæmoglobin from the serum another specimen of the blood should be allowed to coagulate, and when the serum has separated that should be examined separately. The diluted blood should be placed in a small vessel with two plane sides inclined towards each other at an acute angle, so that varying thicknesses of the fluid can be examined.

Either ordinary daylight or a lamp can be used, and

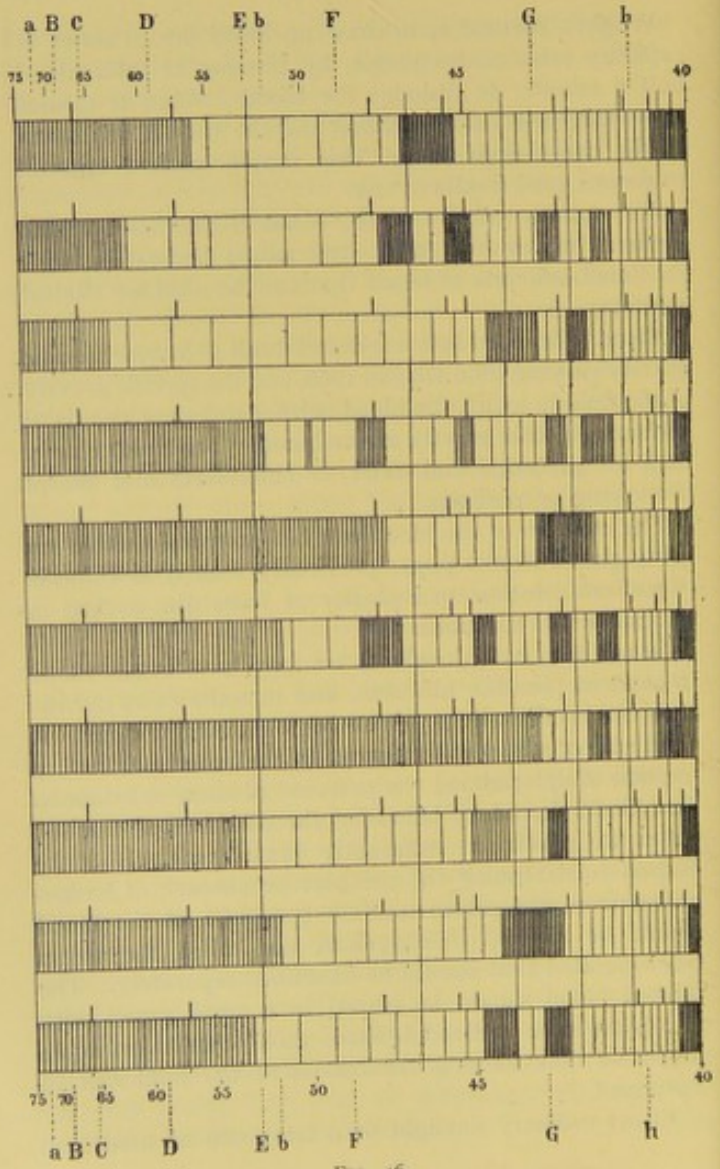


FIG. 46.

the spectrum should first be focussed as sharply as possible and the slit closed as much as is convenient to bring out Fraunhofer's lines distinctly.

The more important spectra are that of oxyhæmoglobin and reduced hæmoglobin, which can be readily obtained from the same specimen either by shaking up with air to oxidise or reducing by the addition of ammonia sulphide.

Methæmoglobin gives two additional lines, as seen in the diagram, and the two lines between D and E are further apart and faint; on the addition of alkali the spectrum changes and becomes more like that of oxyhæmoglobin. Bilirubin is shown by the single faint band between E and F.

The opposite table gives the spectra of hæmoglobin and its derivatives, though many of these are only formed under artificial circumstances and consequently are of little practical clinical value. Some only are found in the urine (fig. 46).

The colouring matter of blood is hæmoglobin, it forms some 90 per cent. of the red corpuscles, and is not found in blood plasma nor when the blood coagulates in the serum. It can, however, be readily removed from the red corpuscles by the addition of water either to the fluid blood or to the freshly dried blood.

Advantage is taken of this property when thick films are made, as in examining for filaria, in order to render a thick film transparent. To prevent the occurrence of this solution in making preparations for the examination of thin films "fixing agents," such as alcohol, perchloride of mercury, formalin solution, or vapour and heat, are employed.

Different specimens of blood vary in power of retaining their hæmoglobin. Though distilled water will remove the hæmoglobin completely from the corpuscles in fluid blood, saline solutions over a certain strength will

not remove it. The resistance of "tonicity" of the blood corpuscles is measured by the strength of saline solution, which is just sufficient to prevent the solution of the hæmoglobin. Such a solution is said to be "isotonic." Normal saline solution ·75 per cent. is sufficient to prevent the solution of hæmoglobin in most bloods, and by using a series of solutions differing by ·02 per cent. and dropping a drop of blood in each and shaking and allowing to stand, we can arrive at a point when the strength of the solution is such, that even when left to stand for some hours no appreciable solution of hæmoglobin takes place.

The strength of that solution gives the isotonic strength, which is the measure of the resistance of the blood, normally 0·46 to 0·48 per cent. A less accurate but more convenient method is to mix a measured amount of the blood with a measured amount of a solution, such as a 3 per cent. solution, which is well above the isotonic strength, and add gradually measured amounts of water till the solution of the hæmoglobin takes place; from this the strength of the solution which just causes solution can be calculated.

Wright's tubes with the air and mixing chamber are convenient for the purpose. The tonicity of the blood is of considerable importance, as a decrease in the tonicity often precedes a hæmolytic attack, such as occurs in blackwater fever, and persons whose blood is of a low degree of tonicity should not be allowed to live in countries where this disease is endemic.

These methods assume that the red corpuscles in the blood are all equally isotonic or nearly so. This is not the case, as even in healthy blood an occasional corpuscle will be found that will be decolourised in a stronger solution of salt than the others, and also a few will retain their hæmoglobin when all the others are decolourised. With healthy blood the great majority of the corpuscles

are equally isotonic or nearly so, but with other bloods a much larger number are markedly less isotonic than the average, and the differences as regards tonicity of the corpuscles are greater.

To determine the range of variation of tonicity in the corpuscles the blood should be first well diluted with a strong salt solution, 4 per cent., which is hypertonic to all corpuscles. The mixing can be done in one of Wright's tubes. The tube is allowed to stand and the corpuscles will fall to the bottom, and can then be expelled by heating the air chamber into a clean watch glass.

Hanging drop preparations of this blood diluted with one, three or seven parts of distilled water will be equivalent to 2 per cent. and 1 per cent. and ·5 of salt solution. An examination of these hanging drops will show if any considerable proportion of the corpuscles have lost their hæmoglobin. If none or very few are decolourised with the 1 per cent. salt solution the remainder of the red corpuscles in the watch glass in 4 per cent. salt solution should be diluted with three parts of distilled water in one of Wright's tubes and well mixed in the mixing chamber. The fluid can be expelled into a clean watch glass and a series of dilutions, as hanging drops, made. One part diluted with one of water will give ·5 per cent., with two of water ·33 per cent. Two parts of the diluted blood with one of water will give ·66 per cent., and so on. In this manner, by examining a series of these hanging drops and determining the proportion of the "shadow" corpuscles which can be easily seen with an oil immersion if the light is cut off, the proportion of corpuscles of lower tonicity than these solutions can be determined.

When the hæmoglobin is dissolved in the serum the blood is said to be "laked." Dissolved hæmoglobin is found in the serum in acute hæmolytic processes, but appears to be rapidly removed either by the hepatic

or renal cells, or deposited in the subcutaneous tissues. The yellow tint of skin and conjunctiva in some diseases which simulate jaundice is of this nature and is called hæmatogenous jaundice. The yellow tinge round old bruises is due to the solution of the hæmoglobin in the extravasated blood.

Normal blood serum is "hypertonic," that is, not only is it sufficient to prevent the solution of hæmoglobin from the red corpuscle, or isotonic, but considerably above that strength. This excess of tonic value is not simply due to the amount of salts. It varies considerably and is estimated by determining the dilution with water required to render it isotonic as regards normal red corpuscles. This can be determined either by using a series of dilutions and dropping (with distilled water, or as a series of hanging drops in Wright's tubes) blood into each, or by diluting to a known extent a mixture of blood and serum.

CHAPTER VII.

Biting-mouthed, Noxious and Parasitic Diptera.

By Fred. V. Theobald, M.A.

The order of two-winged flies or *Diptera* have considerable importance in connection with man and animals and disease.

Diptera are harmful to man and animals in the following ways :—

(1) By biting.

(2) By living as parasites during their larval stage either (*a*) internally (= internal myiasis) ; or (*b*) externally (= external or cutaneous myiasis).

(3) By carrying disease germs, either (*a*) as direct agents, or (*b*) as secondary hosts for parasites.

(1) There are great numbers of diptera which cause considerable annoyance to man by their bites. The virulence of these bites depends on three factors : (*a*) The presence or absence of poisonous saliva produced by the insect and injected into the wound formed by the insect's mouth ; (*b*) the state of the blood in the person bitten ; and (*c*) the state of the insect's piercing organ in regard to cleanliness.

Certain species are noted for their virulent bite ; such insects apparently secrete a poisonous or irritating saliva. Whether the virulence of flies' saliva varies at different times is not known, the dissimilar effects produced by any particular species upon man at different periods and upon separate individuals may be due to the state of

the human being's blood. There is no doubt that at certain times biting insects are more venomous than at others.

Diptera feed both by night and by day; as a rule each species, often each family, has its particular feeding time. The gadflies (*Tabanidæ*), for instance, only feed during the day; *Culicidæ* of the genera *Culex* and *Mansonia* by day and by night; *Anophelina* chiefly but not exclusively by night. Fleas or *Pulicidæ* are almost exclusively nocturnal.

(2) *Parasitism* is fairly common in this order of insects and man is frequently the host. Human dipterous parasites are nearly always found as such in their larval state. There are some notable exceptions in which the adult is the parasite, as the Jigger Flea (*Sarcopsylla penetrans*, &c.). The larvæ of diptera parasitic in man and in animals produce what is technically called *Myiasis*. We find these parasitic larvæ situated internally (internal myiasis) and externally, or under the skin (cutaneous myiasis). The internal position taken up by the larvæ in man is usually the alimentary canal. Grubs such as the horse-bots (*Gastrophilus equi*) may live and develop in the stomach and intestines of the horse, the horse forming a definite host, but they exist in the intestines of man, as the *Anthomyia* larvæ, by chance occurrence and not normally. There are no known dipterous larvæ which live and develop only in man's intestines. Cases of internal myiasis in animals are common, in man rare.

Cutaneous myiasis is much more abundant in man. The eggs of various diptera are deposited on sores and wounds, and the grubs feed in such places (*Lucilia* and *Calliphora*), or the larvæ may live under the skin (*Dermatobia*), or even penetrate the organs of sight. External myiasis does not only refer to the skin but includes cases of insect invasion of the external openings of the body, such as in the nose, ears, vagina, rectum. &c. (Screw-worm, *Compsomyia macellaria*).

Cases of internal myiasis require the most careful attention, as diptera may deposit not only eggs but living young on fæces directly they are voided and these maggots may be thought to have been passed per anum. There are, however, well-authenticated cases of internal myiasis.

(3) Diptera often feed indiscriminately upon man and animals. In this way a biting fly may carry germs of some disease from animal to man, such, for instance, as anthrax, or from man himself to a fellow creature. Another source of infection of disease in man in which diptera play a prominent part is not due to biting diptera alone, but to germs being carried from fæcal matter in latrines, &c., by all kinds of carrion and foul-feeding flies, to man's food and drink (typhoid fever, &c.).

The important *rôle* played by diptera as intermediate hosts of human parasites such as the malarial *Hæmamœbidæ* and the *Filariæ*, is mainly if not exclusively carried out by the *Culicidæ*, or mosquitoes.

The actual specific identification of obnoxious biting and disease-carrying diptera must be left to specialists, but it is of use for the medical practitioner to be able to detect the more important families and genera.

The families and some of the chief obnoxious genera in each are briefly specified in this chapter.

Characters and Structure of Diptera.

The true flies or diptera undergo a complete metamorphosis. They are either provided with two wings or are apterous (*vide* figs. 81, 82). The posterior wings are represented by a pair of club-shaped processes, the "*balancers*," or "*halteres*." The head, thorax and abdomen are distinct. The head is very variable in shape. There are usually two large compound eyes, and ocelli may be present. The antennæ are very variable and present important characters; the number of segments vary.

In the *Nematocerous Orthorrhapha* (*vide* p. 146) they vary from eight to sixteen or more (fig. 47); the two basal ones form the *scape*, the remainder the flagellum; when composed of three segments, as in the *Brachycerous Orthorrhapha*, the third may be either simple or complex (*i.e.*, made up of a number of annuli). The last segment terminates in a simple or plumose bristle (*arista*) or a style (fig. 48).

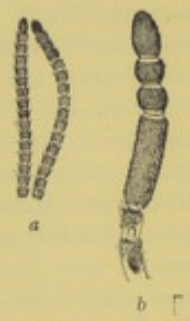


FIG. 47.—ANTENNÆ OF *a*, Mycetid; *b*, Tabanid.

FIG. 48.—ANTENNA (*Brachycerous*). (After Wandolleck.)

The mouth is suctorial and is in some adapted for piercing (*Culicidæ*, *Tabanidæ*, &c.) (figs. 49, 50, &c.). The normal mouth-parts are (1) the *labrum* or upper lip (fig. 49, *f*), (2) the mandibles (fig. 50, *b*), (3) the maxillæ, (fig. 50, *c*), (4) the lower lip or *labium* (fig. 49, *a*), and (5) the hypopharynx (fig. 49, *d*). Jointed appendages, the maxillary palpi (fig. 49, *g*), also are present; the labial palps occur as the so-called *labellæ* (fig. 40, *b* and fig. 50, *c*). The form varies in each group. The labium is more or less fleshy and acts as a sheathing organ; the labrum is often much elongated, and so is the hypopharynx or tongue; the latter and the labrum may form a more or less perfect tube.

The space between the eyes is called the *vertex*, that part in front of the eyes the *frons*, the part behind, the *occiput;* the sides the *genæ*, or cheeks; and a process occurs in front, the *clypeus*.

Nearly half the diptera have a peculiar structure in the form of a vesicle on the head, called a "*ptilinum*." This is a bladder-like expansion in front of the head which appears as the fly emerges from the pupa. It serves to rupture the hard shell in which the fly is enclosed. This "*ptilinum*" becomes completely inverted in the mature fly, it being represented externally by a

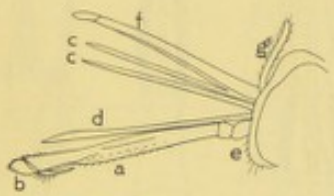


FIG. 49.—MOUTH OF AN *Empis*.

a, Lower lip or labium; *d*, hypopharynx; *b*, labella; *c*, stylets or maxillæ; *f*, upper lip or labrum; *g*, maxillary palp. (After Meinert.)

space, the "*lunula*," under an arched suture extending over the point of insertion of the antennæ.

The head is joined to the thorax by a narrow neck; the back of the head is called the *nape*. The thorax may have all three segments distinct, or the pro- and meso-thorax may fuse; the former is usually small, the latter large; the metanotum is small. The prothorax is most pronounced in the *Nematocera* and forms either two prothoracic lobes or a narrow collar: a portion of the mesonotum is cut off behind by a depressed line, forming the *scutellum*; a transverse suture may sometimes be seen on the mesonotum running across from the base of

the wings, and there is also a prominent groove above the root of the wings, along which there are often characteristic bristles.

The pleuræ or sides are built up of several pieces and lie below the meso- and metanotum, and are known as the (1) *mesopleura* (a space in front of the root of wings

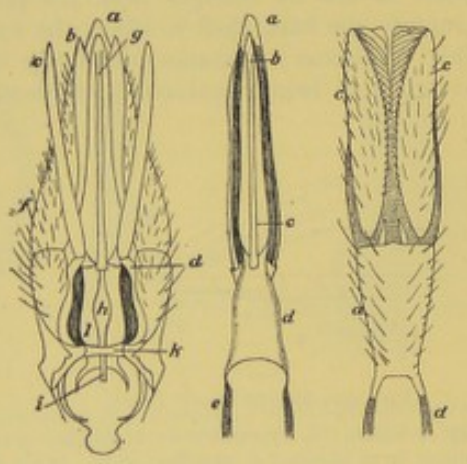


FIG. 50.—MOUTH-PARTS OF *Hæmatopota pluvialis*. ♀ (after Meinert).

a, Labrum; *b*, mandibles; *c*, maxillæ; *d*, basal segment of mouth-parts; *f*, palpi; *g*, hypopharynx; *h*, receptacle for saliva; *i*, salivary duct; *k*, basal part of mouth; *l*, pharynx. In the second figure, *c*, salivary duct. The third figure is the labium—*c*, labella; *a*, scutum; *d*, muscles.

between a suture—the dorsopleural suture that marks off the mesonotum and another suture, the sternopleural, which separates the mesopleura from the sternopleura); (2) the *pteropleura* (a space below the roots of the wings bounded by a suture—mesopleural—which separates it from the mesopleura); (3) *sternopleura* (below sternopleural suture above the front coxæ); (4) *hypo-*

pleura (space over mid and hind coxæ below meta- and pteropleura); (5) *metapleura* (sides of metanotum, to which the halteres are attached).

The *wings* have a variable number of veins, which are both longitudinal and transverse. The figure given here is of a Daddy-long-legs (*Tipula*).

In the centre will be seen a space surrounded by veins —the discal cell (fig. 51, 9). On the fourth longitudinal vein that bounds this cell in front will be seen a short connecting vein—the anterior cross-vein; this always connects the fourth longitudinal vein behind with the third in front, and the cell behind is always the discal cell (9); between the second and third longitudinal veins are the marginal cells. The other cells are shown in the figure.

FIG. 51.—WING OF *Tipula*.

a, Costal vein; *b*, mediastinal vein; *c*, first longitudinal vein; *d*, second longitudinal vein; *e*, third longitudinal vein; *f*, fourth longitudinal vein; *g*, fifth longitudinal vein; *h*, sixth longitudinal vein; *i*, seventh longitudinal vein. 1 and 2, mediastinal cells; 3 and 4, sub-marginal cells; 5, anterior basal; 6, posterior basal; 7, anal; 8, posterior marginal; 9, discal cell. (After Loew.)

The longitudinal veins are known as follows :—

The costal (*a*);
Auxiliary, mediastinal, or subcostal (*b*);
First longitudinal vein (*c*);
Second longitudinal vein or radial (*d*);
Third longitudinal vein or cubital (*e*);
Fourth longitudinal vein or discoidal (*f*);
Fifth longitudinal vein or postical (*g*);

Sixth longitudinal vein or anal (*h*);
Seventh longitudinal vein or axillar rib (*i*);

At the base of the wing is often a scale-like process, the alula, and still nearer the base two smaller processes from the wing, the upper and lower tegulæ. The halteres or balances may be hidden by the alula (*Calyptrate muscids*). The legs are attached to pro-, meso- and metathorax; there are usually distinct ungues or hooks,

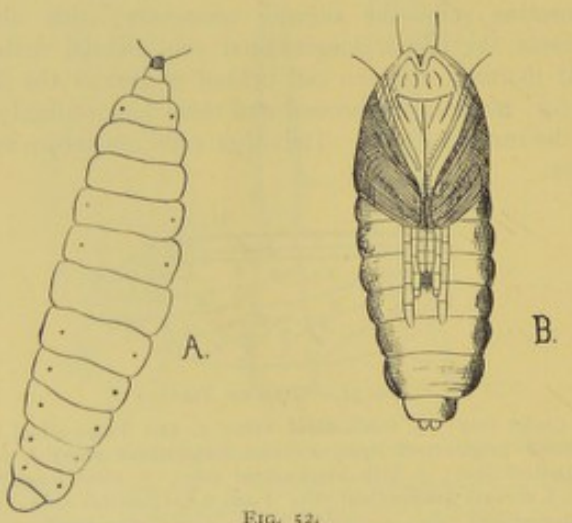


FIG. 52.
LARVA (A) AND PUPA (B) OF *Cecidomyia*. An Orthorrhaphous Dipteron.
(From "A Text-Book of Agricultural Zoology," Theobald.)
(Enlarged 14 times.)

and pulvilli, at the base of the ungues, in the form of two pad-like fleshy cushions, but the latter are often absent in the *Orthorrhapha*. In the *Cyclorrhapha* we find between them the *empodium*, a median appendage in the form of a pad, bristle or spine.

The abdomen is composed of nine segments, but they are not as a rule all shown. The ♂ genitalia or hopopygium are useful as specific characters.

All parts of the body may bear bristles (*chætæ*) which are also important in classification (*chætotaxy*).

The larvæ (fig. 52, A and fig. 73) of all diptera are footless maggots and may (*Orthorrhapha*) (fig. 52, A) or may not have a distinct head (*Cyclorrhapha*) (fig. 73).

The pupæ (fig. 52, B) may be either naked (*Tipulidæ*, *Bibionidæ* and some *Cecidomyidæ*) or enclosed in the hardened larval skin or puparium (*Muscidæ*, *Oestridæ*) (fig. 53).

FIG. 53.—PUPARIUM OF A "SCREW-WORM" (enlarged six times).

The production of living young occurs in some groups (*Cecidomyia*, *Muscids*). In the forest flies and sheep-ticks or keds (*Pupipara*) the young may be born as fully-matured larvæ in a puparium case.

CLASSIFICATION OF DIPTERA.

The classification generally followed now is that formulated by Brauer in 1863. The order is divided into two sub-orders, based mainly upon larval and pupal characters. These two groups are characterised as follows :—

Sub-order 1. ORTHORRHAPHA.—Larva with a distinct head (fig. 52, A); pupa either free or encased in the larval skin (puparium); the larval skin always bursts, for either the exit of the pupa or imago, in a T-shaped opening on the back of the anterior end, rarely in a transverse slit between the eighth and ninth segments. Imago without the frontal lunula and ptilinum.

Sub-order 2. CYCLORRHAPHA.—Larva without any distinct head (fig. 79); pupa always in a puparium (fig. 53); imago always escapes *viâ* a more or less circular opening at the anterior end (fig. 73, C). Frontal lunula always present; ptilinum usually so.

The first sub-order may be divided into two sections: (1) *Nematocera*, (2) *Brachycera*. The *Nematocera* have the antennæ with never less than six joints, usually long and filiform (fig. 47, A), and the palpi three, four or five-jointed. The *Brachycera* have short, three-jointed antennæ (fig. 48), and short, two-jointed palpi. The following families are included in the Orthorrhaphous Nematocera: *Cecidomyidæ* (gall midges), *Mycetophilidæ* (fungus gnats), *Chironomidæ* (midges), *Culicidæ* (mosquitoes), *Bibionidæ* (fever flies), *Simulidæ* (sand-flies), *Psychodidæ* (owl midges), *Blepharoceridæ*, *Ptychopteridæ*, *Tipulidæ* and *Limnobiidæ* (daddy-long-legs). In the Brachycerous section we get the *Lonchopteridæ*, *Stratiomyidæ* (chameleon flies), *Acanthomeridæ*, *Leptidæ*, *Xylophagidæ*, *Tabanidæ* (gadflies), *Nemestrinidæ*, *Acroceridæ*, *Asilidæ* (robber flies), *Mydaidæ*, *Bombylidæ*, *Empidæ*, and *Dolichopodidæ*, &c.

The second sub-order, *Cyclorrhapha*, is also divided into two sections: (1) *Aschiza*, (2) *Schizophora* (*Muscidæ*). The *Aschiza* have the antennæ composed of not more than three joints and an arista, the latter not terminal; the front of the head without any definite arched suture over the antennæ, but frequently with a minute area of different colour or texture there. (*Syrphidæ*, or Hover-

flies, *Conopidæ*, *Pipunculidæ*, *Phoridæ* and *Platypezidæ*). The *Schizophora* have three-jointed antennæ with an arista. In the section *Calyptratæ* the fold over the antennæ is well marked and the halteres are covered with a squama; in the *Acalyptratæ* the halteres are exposed, head and antennæ vary, but cannot be confused with Orthorrhaphous Brachycera because of the less complex veined wings. (*Anthomyidæ*, *Tachinidæ*, *Sarcophagidæ*, *Muscidæ*, *Oestridæ*.) The Fleas, or *Pulicidæ*, are considered to be Diptera and are placed in sub-order *Aphaniptera* by some authors and they are included here as Diptera. They are all wingless and have piercing mouths.

ORTHORRHAPHA—NEMATOCERA.

Family CECIDOMYIDÆ (Gall Midges).—Small, slender flies with long antennæ, with bead-like segments; proboscis short, elongated in one genus only. Abdomen composed of eight segments. Wings usually hairy; no alula; never more than five longitudinal veins, usually only three, the first, third and fifth; fourth and sixth may be present. Costal vein encloses entire wing; fifth vein

FIG. 54.—WING OF A *Cecidomyia*.

forked; only one basal cell. Larvæ all vegetable feeders or inquilines; most produce galls. A few live as parasites in society of plant lice. Larvæ (fig. 52, A) with fourteen segments and possess an "anchor process" under the head end of body. The proboscis is elongated in the genus *Clinorrhyncha* (Loew), and directed downwards.

They are often injurious to crops, but are exceptional in causing annoyance to man by biting.

Family CULICIDÆ (Mosquitoes).—Proboscis elongated for piercing. Eyes reniform; ocelli wanting. Antennæ usually plumose in the ♂ (except *Sabethes*, *Wyeomyia*, &c.). Thorax with large mesothorax, narrow scutellum, rounded metanotum. Abdomen composed of eight segments. Wings (figs. 55 and 56) with six longitudinal

FIG. 55.—WING OF *Anopheles maculipennis*.

veins, exclusive of the sub-costal, and two fork-cells; veins clothed with scales; costal vein continued round the border of the wing, fringed with scales. Head, thorax and abdomen usually but not always scaly. Palpi

FIG. 56.—WING OF A *Culex*.

short or long in the ♀ and ♂. The ♀'s mostly blood-suckers. The majority of mosquitoes come in this family. The larvæ and pupæ are aquatic. The family is divided into the following sections:—

Proboscis long.	
Palpi long in ♀, long in ♂	*Anophelina*.
Palpi short in ♀, long in ♂.	
Metanotum nude.	
Fork-cells long	*Culicina*.
Fork-cells short	*Megarhinina*.
Metanotum scaly	*Joblotina*.
Palpi short in both sexes	*Ædeomina*.
Proboscis short	*Corethrina*.

All the genera except *Corethra* and *Mochlonyx* are blood-suckers.

Family BLEPHAROCERIDÆ.—These little flies have broad wings and long legs. The proboscis is elongated, and the females in some species (*Curupira*) are blood-suckers. The thorax has a distinct transverse suture. The hind legs are longer than the front ones and there are no pulvilli. The broad wings are quite bare, there is no discal cell, they are iridescent, and have a secondary set of fine network of veins. They perform aërial dances like midges, especially near the spray of waterfalls. The larvæ live in rapidly-running water fixed to stones by suckers. Some forms of larvæ (*Curupira*) are composed of only six or seven segments, with widely projecting side lobes and small tracheal gills near the suckers. The pupæ are flattened, inactive, and enclosed in a semi-oval shell, the anterior end having horny erect breathing tubes and suckers on the ventral surface.

Family CHIRONOMIDÆ (Midges).—This family includes the majority of midges which are frequently taken for *Culicidæ* or mosquitoes. They are all small, delicate, gnat-like flies, with small head, partly concealed by the cowl-like thorax. The antennæ in the ♀ are thread-

FIG. 57.—WING OF *Chironomus*.

like and composed of from six to fifteen segments; in the ♂ they are densely plumose. Ocelli wanting or rudimentary. Proboscis short. The oval thorax has no transverse suture, is bare, and projects more or less over the head. The long, narrow abdomen is composed of eight segments and is often semi-transparent

and pilose. The legs are slender and rather long and not spinose. The wings (fig. 57) are narrow, long, and bare or hairy, never scaly; the anterior veins darker than the rest; the sub-costal vein complete but small; second longitudinal vein small or wanting; third longitudinal vein sometimes forked close to its origin, the upper branch often rectangular; fifth long vein forked, sometimes the fourth; the costal vein always ends near the tip of the wing.

FIG. 58.—A *Ceratopogon*.

Great numbers of this family occur in all parts of the world. The members of one genus (*Ceratopogon*) (fig. 58) bite severely. They often occur in swarms, dancing in the air. When at rest they wave their forelegs in the air. *Ceratopogon* occur in most countries. They are known as "punkies" or "no-see-um," and cause great annoyance by their bites.

The larvæ of *Chironomidæ* are mainly aquatic and worm-like, often red in colour, and the pupæ are active; they also live in damp earth and in decaying vegetation. Those of *Ceratopogon* and allied genera in

the sap of trees, under fallen leaves, and in decaying vegetation, or are aquatic, and are long, slender, delicate, whitish creatures. In the genus *Ceratopogon* the dorsum

FIG. 59.—WING OF *Ceratopogon*. (After Leonardi.)

of the thorax is not produced over the head; the palpi are four-jointed; the wings are usually spotted (figs. 58 and 59).

Family PSYCHODIDÆ (Owl-midges).—Small, densely hairy, thick-set insects. Proboscis usually short, but in one genus (*Phlebotomus*) it is long and horny; palpi

FIG. 60.—*Phlebotomus*, sp.
(From Giles' "Gnats or Mosquitoes.")

hairy and composed of four segments. The short abdomen is composed of six to eight segments, hairy. The legs are often short and densely hairy and the claws small. The wings are broad and when at rest lie roof-shaped over the body; they are densely covered with long hairs and are fringed with hairs; neuration mostly composed of longitudinal veins; the first longitudinal

vein near the costa; second arises near origin of first and is usually twice forked; third vein simple; fourth forked; fifth, sixth and seventh usually distinct, the latter sometimes wanting. These small flies can at once be told by their moth-like appearance. They run well, but their flight is weak. Owl-midges frequently occur on windows and in out-buildings. The genus *Phlebotomus* bites severely. The larvæ live in stagnant water and decaying vegetation. They are cylindrical and have a short terminal breathing tube. The inactive pupæ have two long tubular stigmata.

Family SIMULIDÆ (Sand-flies).—Usually called sand-flies, black flies, brulots, buffalo and turkey gnats, and

FIG. 61.—WING OF *Simulium*.

sometimes mosquitoes. All small with oval thorax devoid of any suture. Cylindrical abdomen composed of seven or eight segments. The eyes are holoptic in the ♂, and there are no ocelli. The ♂ is darker and more velvety than the ♀. The short antennæ are composed of ten or eleven segments, the two basal ones distinct, the rest closely united. Palpi composed of four segments, the basal joint short, the next two equal, the last longer and narrowed. The legs short, thick; femora broad and flat. Wings (fig. 61) large and broad, the anterior veins thickened, remainder delicate; the sub-costal terminates in the costa about half the length of the wing; first and third longitudinal veins lie close together; fourth vein forked nearly opposite the anterior

cross-vein; forks terminate near the tip of the wing. Proboscis short with strong horny lamellæ, consists of two resisting bristles for puncturing, and on its sides two maxillary four-jointed palps. These small flies bite very severely and cause much annoyance. They especially attack the eyes, nostrils and ears of both animals and man. Sand-flies occur in all climates. The larvæ are all aquatic and live in rapidly flowing water; they attach themselves to stones, plants, &c., and form elongated cocoons, open above. They are soft-skinned, with thickened ends, a cylindrical head, and on the first segment a prominence with bristly hooks, and the end of the abdomen with several appendages, by which the larvæ attach themselves. The pupæ have the anterior end of the body free and from it pass out a number of thread-like breathing tubes. The flies are accused of propagating anthrax and septic diseases. Their punctures give rise to severe inflammation and depilation in animals.

ORTHORRHAPHA BRACHYCERA (Antennæ short).

Family TABANIDÆ (Gad-flies).—This family includes a number of genera, the popular names being gad-, breeze- or horse- flies, brimps and sneggs. They are mostly large

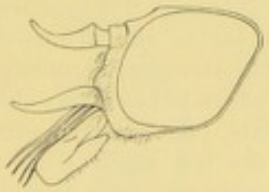


FIG. 62.—HEAD OF *Tabanus*.

and stout; the head (fig. 62) large; eyes very large, contiguous in the ♂, the upper facets larger than the

lower, usually with green and violet markings when alive. The antennæ composed of three segments, third segment composed of six to eight rings; no stylet. The proboscis prominent, often greatly elongated, fleshy, with pointed horny processes; the ♀ with six, the ♂ with four stylets; the former only is sanguineous. Palpi two-jointed, the second joint large. The abdomen is broad, often flattened, never slender, composed of seven segments (*vide* fig. 64). The legs are rather thick, mid-tibiæ always with spurs; tarsi with three membranous pads at the tip. There are never any bristles. The third longitudinal vein forked. Two submarginal and five posterior cells present; anal cell closed at or near margin of wing. Tegulæ large.

Fig. 63.—Wing of a *Tabanus*.

Mostly large flies which occur during hot weather and have remarkable powers of flight. The bite of the ♀ is often severe. The eggs are spindle-shaped and dark, and are laid on leaves and stems of plants and on water plants. The larvæ are carnivorous and feed upon snails, insect larvæ and also roots; elongated, composed of eleven segments, jointed, often with retractile fleshy protuberances; the last segment has a breathing pore, or the last two segments may form a breathing tube. The pupæ are free, and live in earth and water.

The worst biting species are found in the following genera: *Pangonia*, *Chrysops*, *Hadrus*, *Hæmatopota*, *Therioplectes*, *Atylotus* and *Tabanus*.

een and violet markings when
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s to eight rings; no stylet. The
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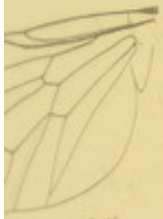

Wing of a *Tabanus*.

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abanus.

Fig. 64.—*Tabanus bovinus.*

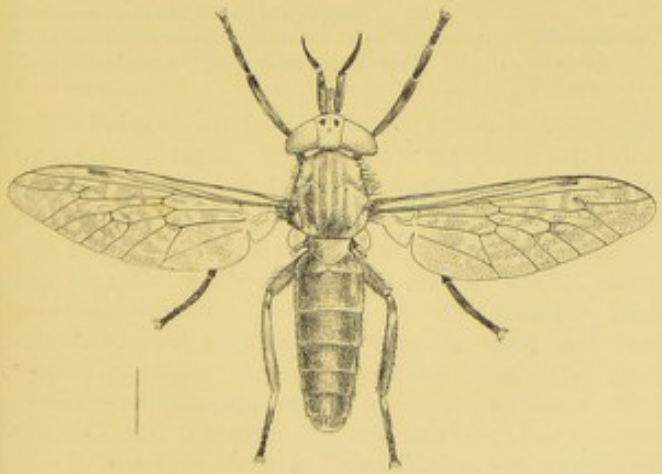


Fig. 65.—*Hæmatopota pluvialis.*

There are two sections, distinguis

Hind tibiæ with spurs at the tip .....
Hind tibiæ without spurs .....

The following characters separate

genera :—

Pangoniinæ.

Third joint of antennæ eight-ringed, the
slightly the longer; the fourth post
apex; proboscis often very long.....
Third joint composed of five rings, the first
is much longer than the following; t
joint of antennæ as long as the first; w
dark areas; three ocelli; brilliant
purple lines and spots .....

Tabaninæ.

Third joint of antennæ without or with a
mentary basal process; thorax and
with iridescent tomentum; tibiæ dilate
Thorax and abdomen without iridescen
tum; front of ♀ as broad as long .....
Third joint of the antennæ with well-
basal portion.
First antennal joint short; body broad.
Eyes pubescent, small ocelligerous tubercle
Eyes pubescent, but no ocelligerous tubercl
Eyes bare.....

The Pangonias are found in woodl
their flight is rapid. The proboscis
gated so that they can pierce thro
An epizooty of anthrax in Pine L
was traced to this genus (*Magnus*)
fig. 64), is world-wide; the
proboscis and the last joint of
annulated and notched in cres
large size render them easily id
Hæmatopota (fig. 65) has no cres
(fig. 66), and the wings (fig. 67)
is also narrower than in *Tabanus*
hyaline spots.

There are two sections, distinguished as follows :—

Hind tibiæ with spurs at the tip	*Pangoninæ.*
Hind tibiæ without spurs	*Tabaninæ.*

The following characters separate the above-mentioned genera :—

Pangoninæ.

Third joint of antennæ eight-ringed, the first ring slightly the longer; the fourth posterior cell open; proboscis often very long.....	*Pangonia.*
Third joint composed of five rings, the first of which is much longer than the following; the second joint of antennæ as long as the first; wings with dark areas; three ocelli; brilliant eyes with purple lines and spots	*Chrysops.*

Tabaninæ.

Third joint of antennæ without or with only a rudimentary basal process; thorax and abdomen with iridescent tomentum; tibiæ dilated	*Hadrus.*
Thorax and abdomen without iridescent tomentum; front of ♀ as broad as long	*Hæmatopota.*
Third joint of the antennæ with well-developed basal portion.	
First antennal joint short; body broad.	
Eyes pubescent, small ocelligerous tubercle present...	*Therioplectes.*
Eyes pubescent, but no ocelligerous tubercle	*Atylotus.*
Eyes bare.....	*Tabanus.*

The *Pangonia* are found in woods, forests and pastures; their flight is rapid. The proboscis may be greatly elongated so that they can pierce through even thick clothes. An epizooty of anthrax in Pine Island, New Caledonia, was traced to this genus (*Megnin*). The genus *Tabanus* (fig. 64), is world-wide; the short, thick, salient proboscis and the last joint of the antennæ being annulated and notched in crescentic form and their large size render them easily identifiable. The genus *Hæmatopota* (fig. 65) has no crescentic antennal notch (fig. 66), and the wings (fig. 67) overlap; the abdomen is also narrower than in *Tabanus*, and the wings have hyaline spots.

The genus *Chrysops* can usually be told by their wings (fig. 68) being marked with dark areas and their eyes with purple lines and spots. They bite severely and usually attack round the eyes. An example of *Hadrus*

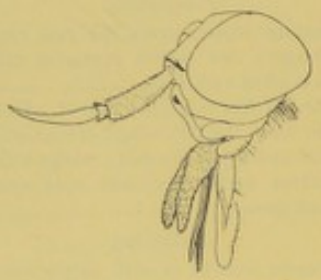


Fig. 66.—Head of *Hæmatopota*.

Fig. 67.—Wing of *Hæmatopota pluvialis*.

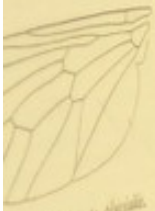
Fig. 68.—Wing of *Chrysops cæcutiens*. (After Leonardi.)

is the Motuca fly (*H. lepidotus*) of Brazil, which causes deep wounds.

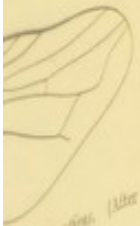
Family Asilidæ (Robber-flies).—Mostly large flies, usually more or less elongated in form, and often thickly

an usually be told by their wings with dark areas and their eyes spots. They bite severely and e eyes. An example of *Hadrus*

Head of *Hæmatopota*.



g of *Hæmatopota pluvialis*.



...myopa cornuina. (After Leonard.)

...lophidatus) of Brazil, which causes

Robber-flies).—Mostly large flies, ...ongated in form, and often thickly

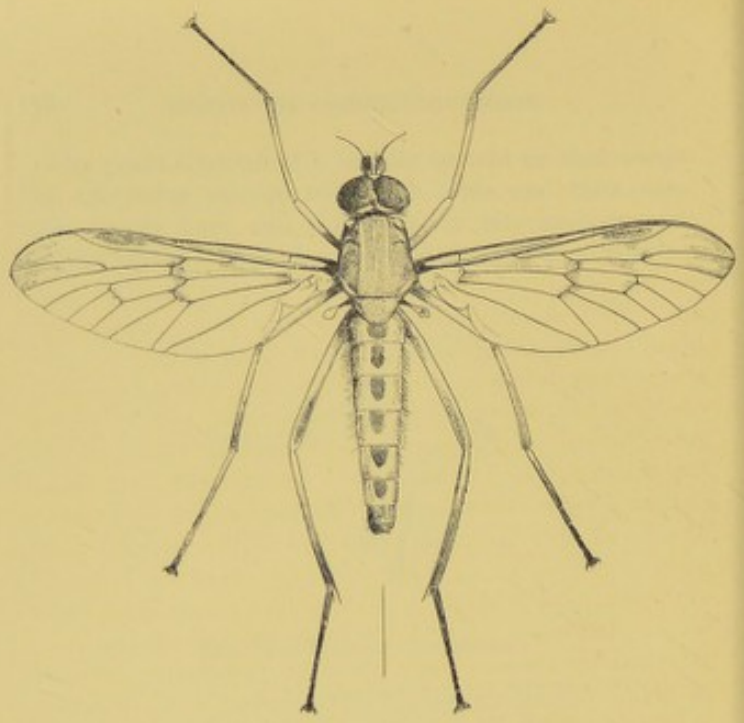


FIG. 69.—*Leptis scolopacea.*

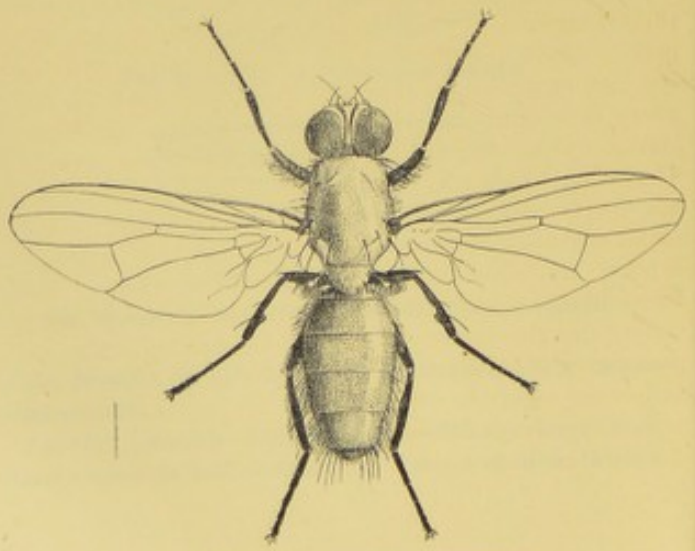


FIG. 70.—*Homalomyia canicularis.*

hairy and with strong bristles. Head broad and short with a freely movable neck; eyes separate in both sexes. Antennæ composed of three segments, the third elongated, generally simple, with or without a terminal style or bristle; style thickened and forming one or two apparent antennal joints. Proboscis firm; upper lip horny, used for piercing; labella not fleshy. Legs strong and bristly. Wings when closed lying parallel over the abdomen; three long basal cells, two or three sub-marginal cells and five posterior cells long; third longitudinal vein forked. These flies usually feed upon insects. Some reach as much as two inches in length. The larvæ live in rotten wood and in the soil and feed upon other larvæ. There do not seem to be any authentic records of these Robber-flies biting man, but some of the larger tropical species do so; animals are also attacked by them. There are over 150 genera in this family.

Family LEPTIDÆ.—This family includes a number of elongated flies of moderate or large size (fig. 69). The veins of the wings distinct, not crowded anteriorly; third longitudinal vein forked, basal cells large; five posterior cells usually present. Third joint of antennæ complex or simple, with or without a terminal or dorsal arista or a terminal style. One genus only (*Symphoromyia*) bites, the rest being predacious upon insects. The section *Leptinæ*, in which the biting genus occurs, has short antennæ with simple third joint, with a terminal or dorsal arista or a terminal style; the proboscis is short and some or all the tibiæ have spines. The larvæ live in the earth and in decaying wood, sand, water, and the nests of wood-boring beetles; they are predacious; usually cylindrical and may have fleshy abdominal legs; the anal segment has a transverse cleft and often two posteriorly directed processes, and two stigmata between them. The genus *Symphoromyia* has

a single spur on the third tibiæ; the third joint of antennæ kidney-shaped, and the arista nearly dorsal.

Family EMPIDIDÆ.—This family is a large one and includes many genera. The flies have a piercing mouth, being all predaceous, feeding upon other insects. Probably some attack man in the Tropics. They are mostly small to moderate-sized species with small head provided with either a short or long proboscis. The proboscis (fig. 48) consists of two stylets (*c*), a hypopharynx (*d*), and an upper (*f*) and lower tip (*a*). The antennæ three-jointed, the first two joints often small, third joint very variable, with or without a terminal arista or style. Abdomen of

FIG. 71.—WING OF *Empis*.

from five to seven segments, male genitalia very prominent. The legs have peculiar structures, the femora thickened and spiny; metatarsi flattened. Neuration (fig. 71) of wing variable; there are three or four posterior cells; the anal cell is closed remote from the border, sometimes wanting, at other times it is closed near the border (*Hilarimorpha*); then the discal cell is wanting. Tegulæ small. The larvæ are cylindrical, with small ventral swellings on the mesothoracic segments; they live in earth and amongst decaying vegetal matter. The pupæ have two points at the anterior end.

CYCLORRHAPHA—ASCHIZA.

This section does not contain any members that bite or annoy man.

The chief family, the *Syrphidæ* or Hover Flies, are noted for the good some of their larvæ do in destroying Aphides.

CYCLORRHAPHA—SCHIZOPHORA.

I. MUSCIDÆ ACALYPTRATÆ.—Mostly small flies with the antennæ composed of three segments bearing a non-terminal bristle; halteres never covered by a squama or basal scale; nervuration of wings simple, few cells.

This group contains a large number of sub-families. None as far as I am aware are annoying to any noticeable extent to man. The following families are of

FIG. 72.—*Dermatobia noxialis.*

economic importance, agriculturally and otherwise: *Chloropidæ*, *Trypetidæ*, *Psilidæ* (as vegetable feeders), *Scatophagidæ* (dung-flies).

II. MUSCIDÆ CALYPTRÆ.—Halteres covered with a squama.

Family OESTRIDÆ (Warble Flies) (fig. 72).—Flies of large size, thick-set, and often very hairy. Mouth small, parts rudimentary; eyes rather small, bare. Head large;

the antennæ small, composed of three segments, more or less hidden ; arista simple or plumose. Thorax broad, with distinct transverse suture. Abdomen short and thick. Legs of moderate length, the hind pair often longer than the rest. Wings with or without markings ; anal cell,

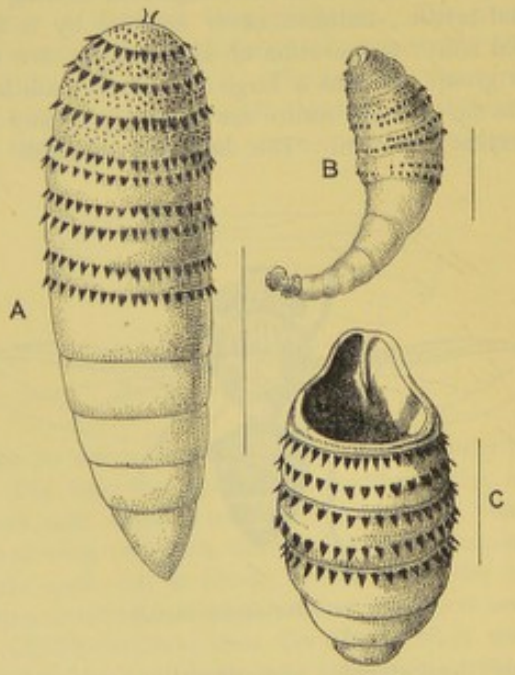


FIG. 73.—*Dermatobia noxialis.*
A and B, Larvæ ("bots"); C, puparium. (After Brauer.)

small discal cell, may be absent. The larvæ or bots (fig. 73, A and B) are provided with circles of spines, two hooked mandibles and anal breathing pores ; parasitic. They live in three ways—(1) under the skin, (2) in nasal and pharyngeal cavities, and (3) in the alimentary canal.

Man as well as animals may be attacked (*Dermatobia*). The larvæ of *Dermatobia* (fig. 73) live under the skin of man, apes, cattle, dogs, &c. In the adult *Dermatobia* the arista is plumose on the upper side and the tarsi slender; the proboscis is bent at the base and is concealed in the buccal cavity; tegulæ large; first posterior cell closed; body hairy. Larvæ club-shaped, slender posteriorly, and surrounded with rows of prickles on the borders of the segments of the apical half. The chrysalis stage is formed in a hard puparium case (c). The common species, *D. noxialis* (Goudot), occurs from Mexico to Brazil, and is known as the "macaw worm," "ura," "torcel," and "moyoquil worm."

Family SARCOPHAGIDÆ (Flesh-flies). — Usually thick-set and of variable size. Abdomen composed of four visible segments with bristles which are confined to the anal end, but sometimes elsewhere. Arista plumose to the middle, *apex always bare*. Some are metallic (*Cynomyia*). Larvæ feed on decaying animal and vegetable matter and may live as parasites in the flesh of animals and in the orifices of man, also in wounds and ulcers. Those of *Sarcophaga* often occur in wounds in man, and are sometimes produced alive. The larvæ are rounded, and thin anteriorly; abdominal segments distinct, each with a circle of spines; mouth with two curved mandibles; posterior stigmata placed in a deep cavity, and there are two pointed anal swellings. The pupa lies in a brown oval puparium.

The genus *Sarcophaga* (Meigen) has the first posterior cell open; the tibiæ with a few bristles; the mid and posterior cross-veins nearly in the same line.

Sarcophaga carnaria, the common British flesh-fly, may be taken as an example.

Cynomyia (Desvoidy) has a metallic abdomen and the tibiæ with short hairs.

Cynomyia mortuorum is a bright blue fly about the size

of a blow-fly, and like it lays its eggs in decaying animal matter, and may possibly do so on wounds.

Sarcophila (Rondani), like others in the *Sarcophagidæ*, are viviparous. The females deposit their larvæ in wounds in animals and man.

The genus *Auchmeroyia* contains specimens that produce cutaneous myiasis, such as the Natal maggot-fly (*A. depressa*).

The larvæ of the genus *Ochromyia* are also parasitic under the skin of animals and man—Cayor or Senegal fly (*O. anthropophaga*).

Family MUSCIDÆ (House Flies, Tsetse Flies, &c.).—A large family, easily told from the former by the arista being plumose at the tip (now and then it is bare), there are no bristles on the abdomen except at the tip, and the first posterior cell is very narrow). The eyes of the ♂ contiguous, bare or hairy in both sexes. Abdomen composed of four visible segments. This family contains the house fly (*Musca*), blue- and green-bottle flies (*Lucilia* and *Calliphora*), stable or "stinging flies" (*Stomoxys*), horse-flies (*Hæmatobia*), and tsetse flies (*Glossina*). The larvæ are variable and live in decaying vegetation, in decaying animal matter and fæces; others, as the screw-worm (*Compsomyia*), as parasites in animals and man: so also may *Calliphora* and *Lucilia*. The *Stomoxyinæ*, which include the stable fly, tsetse fly and the horn fly, have elongated, piercing proboscis, and are blood-suckers.

The following characters will separate the more important genera :—

Proboscis long, used for piercing; palpi shorter than proboscis	*Stomoxys.*
Palpi nearly as long as proboscis	*Hæmatobia.*
Proboscis very long, straight	*Glossina.*

Proboscis short, not adapted for piercing; arista plumose on both sides; curvature of fourth vein angular; mid tibiæ without bristles on inner side;

Fig. 74.—Lucilia

Fig. 75.—Compsomyia

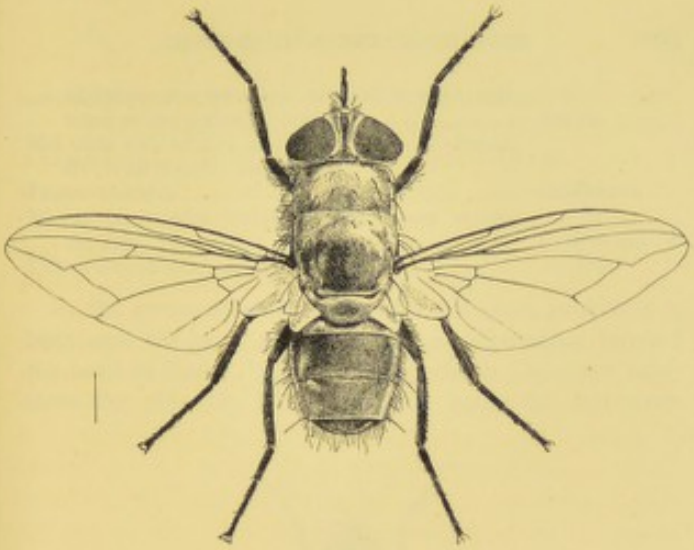


FIG. 74.—*Lucilia cæsar.*

FIG. 75.—*Compsomyia macellaria.*

abdomen non-metallic; blackish sp
more or less yellowish markings.....
Mid tibiæ with bristles on the inner side
&c., with metallic colours.
Thorax blackish.....
Thorax black with whitish stripes, m
metallic.....
Thorax unicolorous, metallic .....

In the genus *Stomoxys* the sole
bent near its base, is horizontal
the head in front. The typical sp
brus (fig. 76), very like a comm

Fig. 76.—*Stomoxys c*

spotted with grey and black. Th
fine and pricks severely. In the
proboscis is similar, but the palp
Hæmatobia serrata, the "horn fl
causes much annoyance to cattl
in Great Britain it seems har
Glossina the proboscis is long an
bite animals and man. Glossina
tsetse fly, carries the blood para
horses and other animals—the

abdomen non-metallic; blackish species with more or less yellowish markings..... *Musca.*
Mid tibiæ with bristles on the inner side; abdomen, &c., with metallic colours.
Thorax blackish..... *Calliphora.*
Thorax black with whitish stripes, more or less metallic..... *Compsomyia.*
Thorax unicolorous, metallic ..... *Lucilia.*

In the genus *Stomoxys* the solid, elongate proboscis is bent near its base, is horizontal and extending beyond the head in front. The typical species is *Stomoxys calcitrans* (fig. 76), very like a common house fly, but more

FIG. 76.—*Stomoxys calcitrans.*

spotted with grey and black. The proboscis is hard and fine and pricks severely. In the genus *Hæmatobia* the proboscis is similar, but the palpi at once separate it. *Hæmatobia serrata*, the "horn fly" of North America, causes much annoyance to cattle and bites man, but in Great Britain it seems harmless. In the genus *Glossina* the proboscis is long and straight. These flies bite animals and man. *Glossina morsitans* (fig. 77), the tsetse fly, carries the blood parasite (*Trypanosoma*) of horses and other animals—the Nagana or fly disease.

The bites, although severe, are not dangerous to man, unless by carrying similar germs. This genus produces its larvæ full grown, the larvæ changing to pupæ at once.

FIG. 77.—*Glossina morsitans.*

Genus *Lucilia* (fig. 74), the so-called greenbottle flies,

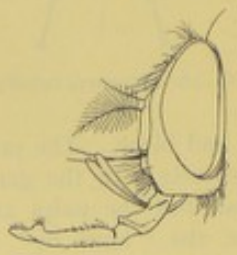


FIG. 78.—HEAD OF *Lucilia cæsar.*

have a soft proboscis (fig. 78). They are all metallic and the abdomen is short and round; the antennæ to the third segment are quadruple the size of the second. The ova and larvæ are often deposited on wounds and

ulcers in animals and man (*L. sericata*). This species causes the well-known "maggot" in sheep.

Genus *Compsomyia*.—This genus also contains metallic flies which differ from *Lucilia* in that the thorax is striped. The screw-worm fly (*C. macellaria*, fig. 75) is found in North and South America and the West Indies, but does not attack man farther north than Kansas.

Family ANTHOMYIDÆ.—These are mostly moderate-sized, dull-coloured flies, resembling the common house fly. The arista is plumose, pubescent or bare. Abdomen composed of four or five segments; sometimes there are no bristles on the body, but they are usually present. The first posterior cell of the wings broadly open; tegulæ of considerable size. Male eyes contiguous or nearly so. It is closely connected on one hand with the *Muscidæ* and on the other with the *Sarcophagidæ*. None are metallic. The open first posterior cell is the chief character. The following genera have been connected with man either as parasites or by causing other annoyance, viz., *Hydrotæa* (Desvoidy), *Homalomyia* (Bouche), and *Hylemyia* (Desvoidy).

They may be told as follows:—

Eyes of ♂ close together; tegula large;* fore femora of ♂ with processes (tubercles, &c.) below; arista always somewhat pubescent; eyes bare; black or blue-black, and pilose ..... *Hydrotæa* (Desvoidy)

Eyes of ♂ close together, bare; tegula large; abdomen nearly bare, unspotted; head almost composed of eyes; antennæ short, third joint elongated; arista bare; mid legs of ♂ often with peculiar structures; black and grey ..... *Homalomyia* (Bouche).

Arista plumose; eyes bare; elongated species, grey or black ..... *Hylemyia* (Desvoidy).

The genus *Homalomyia* (fig. 79) has often occurred

* Larger than ante-tegula.

in human beings in the larval state in the intestines, being passed alive in the fæces. Most larvæ in this family are vegetable feeders. They are normally

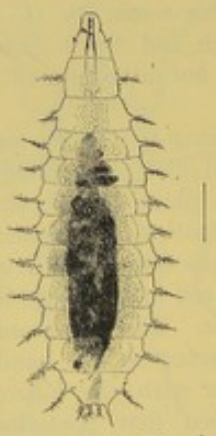


FIG. 79.—LARVA OF *Homalomyia*.

slender and cylindrical, or flat and oval, with four rows of thread-like processes on the segments, and have two mouth hooks. The puparium may be oval or flattened. In *Homalomyia* they have curious branched processes (fig. 79). The genus *Hydrotæa* also occurs in the larval

FIG. 80.—WING OF *Hydrotæa ciliata*.

form in human beings. The characteristic neuration is shown in fig. 80. *Hylemyia* larvæ have also occurred in human excreta, having been passed per anum. Some are dung frequenters and produce living young.

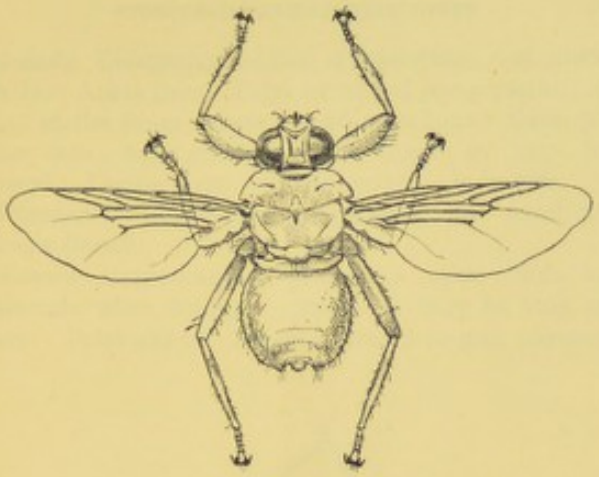


FIG. 81.—*Hippobosca equina* (enlarged four times).

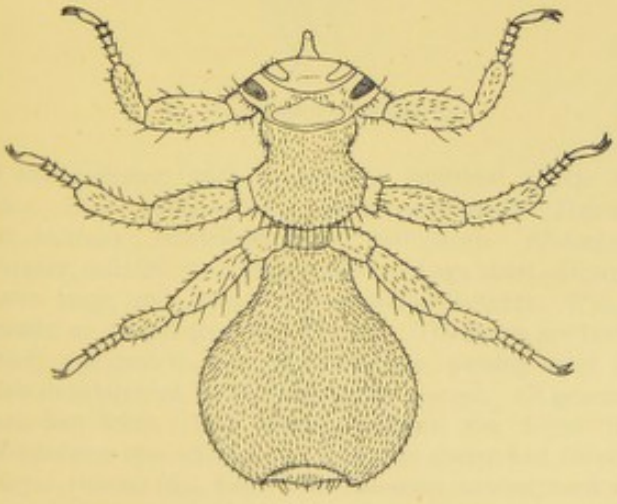


FIG. 82.—*Melophagus ovinus* (enlarged twelve times.)

Family TACHINIDÆ.—Like *A*
bristly. Arista bare. Palpi forme
veins of the wings simple; basal
terior cells; first posterior cells
opened. Tegulæ large. Larvæ p
PUPIPARA.—Blood-sucking, pa
(except *Braula*).
Family HIPPOBOSCIDÆ.—Para
mammals when mature. Probo
sharp. Palpi absent; antennæ pl

FIG. 83.—HEAD OF *H*

of one segment, with or withou
lunes. Eyes round or oval, ofte
flat, leathery; scutellum broad
leathery, inflated, no sutures visibl
claws large and dentate; emp
present or absent (*vide* figs. 81, 8
usually matured in the puparium
their development in the body of
louse-like form. This family e
(*Hippobosca equina*) (fig. 81), and
(*Melophagus ovinus*) (fig. 82). The
elongated, hard, closely-applied

Family TACHINIDÆ.—Like *Anthomyidæ*, but always bristly. Arista bare. Palpi formed of one segment. All veins of the wings simple; basal cells large; three posterior cells; first posterior cells closed or only just opened. Tegulæ large. Larvæ parasitic in insects.

PUPIPARA.—Blood-sucking, parasitic on vertebrates (except *Braula*).

Family HIPPOBOSCIDÆ.—Parasites upon birds and mammals when mature. Proboscis may be long and sharp. Palpi absent; antennæ placed in pits, composed

FIG. 83.—HEAD OF *Hippobosca*.

of one segment, with or without terminal bristle or hairs. Eyes round or oval, often very small. Thorax flat, leathery; scutellum broad and short. Abdomen leathery, inflated, no sutures visible. Legs short, strong; claws large and dentate; empodia distinct. Wings present or absent (*vide* figs. 81, 82). The larvæ are born nearly matured in the puparium case, passing most of their development in the body of the parent. Of general louse-like form. This family contains the forest fly (*Hippobosca equina*) (fig. 81), and the sheep ked (*Melophagus ovinus*) (fig. 82). The proboscis is composed of elongated, hard, closely-applied flaps and an inner tube

between. They mostly live on birds, and now and then these animal pests attack man.

Family NYCTERIBIDÆ.— Found exclusively on bats. Spider-like; no wings. Eyes and ocelli indistinct or wanting. Legs long, femora and tibiæ flattened.

Two other families, *Braulidæ* and *Streblidæ*, occur; the former live on bees, the latter on bats.

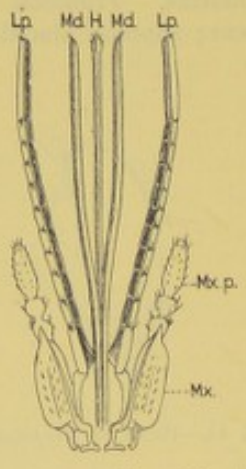


FIG. 84.—MOUTH-PARTS OF A FLEA (*Vermipsylla alakurt*, ♂). (After Wagner.)

h., Median lancet; *lp.*, labial palpi; *md.*, mandibles; *mx.*, maxillæ; *mx.p.*, maxillary palpi.

Sub-order APHANIPTERA (Fleas).—This sub-order contains the group of fleas or *Pulicidæ*. These are all apterous and are provided with a piercing mouth (fig. 84); the body flattened laterally; the head small, scarcely separated from the body; the antennæ are short and thick and lie in pits in the head; the eyes are represented by two ocelli or may be absent. The three segments of the thorax distinct. Abdomen of nine segments. Legs

adapted for jumping, mainly modified in the third pair. The mouth-parts consist of well-developed maxillæ (*mx.*), mandibles (*md.*), and labium (fig. 84). The mandibles are in the form of a pair of elongated styles with serrated edges; the maxillæ have each a palp (*mx.p.*); the labial palpi (*lp.*) are well developed and imperfectly jointed transversely and act as sheaths; in the middle is the unpaired pricking organ, the hypopharynx (*h*). They are all parasitic on man, animals and birds.

The following are human parasites: the common flea (*Pulex irritans*), the alakurt (*Vermipsylla alakurt*), and the jigger (*Sarcopsylla penetrans*). The larvæ are usually free and are found amongst dust and dirt, in humours on the skin of animals, &c. The jigger differs in that the ♀ buries herself under the outer skin of animals and man and the abdomen swells up with the developing eggs. In the Alakurt the female also attacks cattle, it remains attached to its host for many months like a tick, and its body swells.

The genus *Sarcopsylla* has the forehead angular, with a number of tooth-like points. *S. gallinacea* attacks not only fowls but also children. In the genus *Pulex* the eyes are distinct. In *Typhlopsylla* they are absent or indistinct; on the underside of the head are bristles and also on the pronotum and on the abdominal segments. In *Hystricopsylla* the cheeks and clypeus are densely spinose and the eyes absent or rudimentary. Species of *Typhlopsylla* occur in rats, mice, hares and voles.

CHAPTER VIII.

Mosquitoes.

Mosquitoes are the hosts of many parasites and some of those are injurious to man or the lower animals. As these diseases include malaria, at least one of the human filariæ, as well as the *Proteosoma* of birds and the *Filaria immitis*, so fatal to dogs, a good working knowledge of the structure, life-history and modes of classification of these insects is required for tropical work.

Mosquitoes, or *Culicidæ*, belong to the order of dipterous insects. They are characterised by having the anterior pair of wings membranous, whilst the posterior pair are represented by a pair of club-shaped processes known as halteres or balancers.

The insect is divided naturally into three regions: (1) head, (2) thorax, (3) abdomen. To the head are attached the sensory and biting organs, consisting of two compound eyes, two antennæ, two palpi, and a complex suctorial and piercing organ, the proboscis.

To the thorax are articulated a pair of wings, a pair of balancers and three pairs of legs, whilst the abdomen is segmented and terminates in the anus and external organs of generation.

The head, thorax, abdomen and legs are thickly covered with scales in most of the genera, whilst on the wings scales are only found at the edge and on the veins. The character and arrangement of the scales are important points in the differentiation of genera. The absence of

scales on the wings or the presence of scales on other parts of the wings than those mentioned, or the substitution of hairs for scales, are valuable aids in the differentiation of other insects from the *Culicidæ*. The type of venation of the wings and the characters of the cephalic appendages are of value both for identification of the family *Culicidæ*, differentiation of genera, of species, and of sexes.

Scales lose their colour and become too transparent for proper examination unless the specimens are mounted dry, so that for identification of species it is advisable that the mosquities should be mounted dry and so arranged that all the surfaces can be examined. Young mosquitoes are the best to examine or send for examination, as in older specimens many scales are rubbed off and the insects otherwise injured.

The mosquitoes must be killed rapidly, and a cyanide pot is invaluable in this connection, though at a pinch chloroform vapour, formalin, or even tobacco smoke, may be used. The dead mosquito must be mounted without delay as the limbs soon lose their pliability. They should be placed on their backs on a piece of cork felt. A small square or a circle of thin card should be taken and on one side of it the date and place of capture of the mosquito should be written, with a distinguishing number if the name is not known.

The finest entomological pins (No. 20) should be taken up with forceps *near the point*, and the piece of card with the blank side downwards should be placed on a piece of cork felt. The pin still held in the forceps should be pushed through the card. The hold on the pin should then be shifted higher, and the pin pushed still further through the card till about half of it is through. The pin, still held in the forceps with the card transfixed on it, should then be pushed through the thorax of the mosquito. On lifting out the pin the mosquito, which

has been transfixed, will remain on the pin, and on turning the card upside down the legs and wings can with a few touches of a clean needle be arranged so as to be readily visible, and will not hide any part of the back of the insect. A stout pin should then be run through the corner of the piece of card into the cork felt floor of the collecting box. Some powdered naphthaline enclosed in cloth should be placed in the box to prevent insects attacking the specimens.

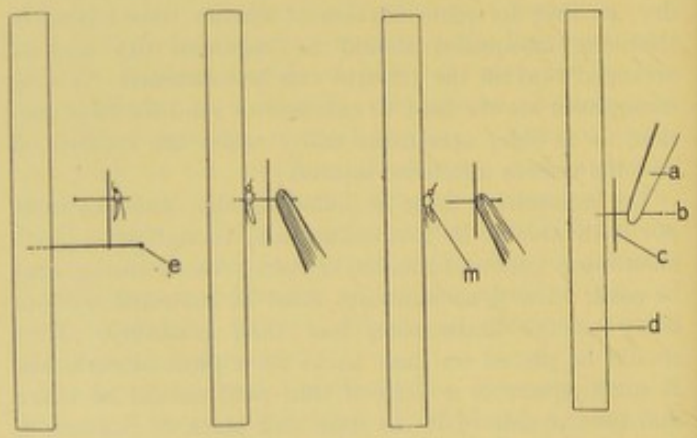


FIG. 85.
a, Forceps; *b*, pin; *c*, disc; *d*, cork; *m*, mosquito; *e*, large pin to carry disc.

To examine such a specimen a low power, one inch or two-thirds of an inch, is required. With such a power the character of the scales on each part of the insect can be examined. The examination should be made by reflected light and the insect so rotated that the part to be examined is horizontal. This can be done best by altering the inclination of the large pin and using a strip of cork felt as a slide (fig. 86). In this way each part of the upper surface can be examined in succession.

To examine the under surface a second mosquito mounted with its back towards the card is required.

The main types of scales found in the *Culicidæ* are represented in the drawing (fig. 87).*

These scales can for descriptive purpose be reduced to the small number of types represented :—

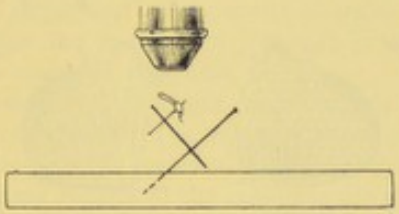


Fig. 86.

(*a*) Broad, flat, spade-shaped or tile-shaped scales.
(*b*) Broad, expanded, asymmetrical scales.
(*c*) Narrow, asymmetrical scales.
(*d*) Narrow, hair-like scales.
(*e*) Narrow curved scales.
(*f* & *g*) Spindle-shaped scales.
(*h* & *i*) Upright fork scales.
(*j*) Long twisted scales.
(*k*) Pyriform scales.

On the wings other types of scales, either lanceolate, long, narrow scales pointed at the free end, or long and narrow and with square free ends, are met with (fig. 91).

Head appendages.—The head appendages can be easily seen in most specimens mounted as described, but for more minute examination it is better to cut off the head and mount it in a shallow cell either as a dry specimen or in glycerine jelly. In this way the parts are not much

*Figs. 87-91 are reproduced by kind permission of the Editor of *The Journal of Tropical Medicine*.

distorted, and if a thin slide be used both sides of the specimen can be examined. Canada balsam can be used,

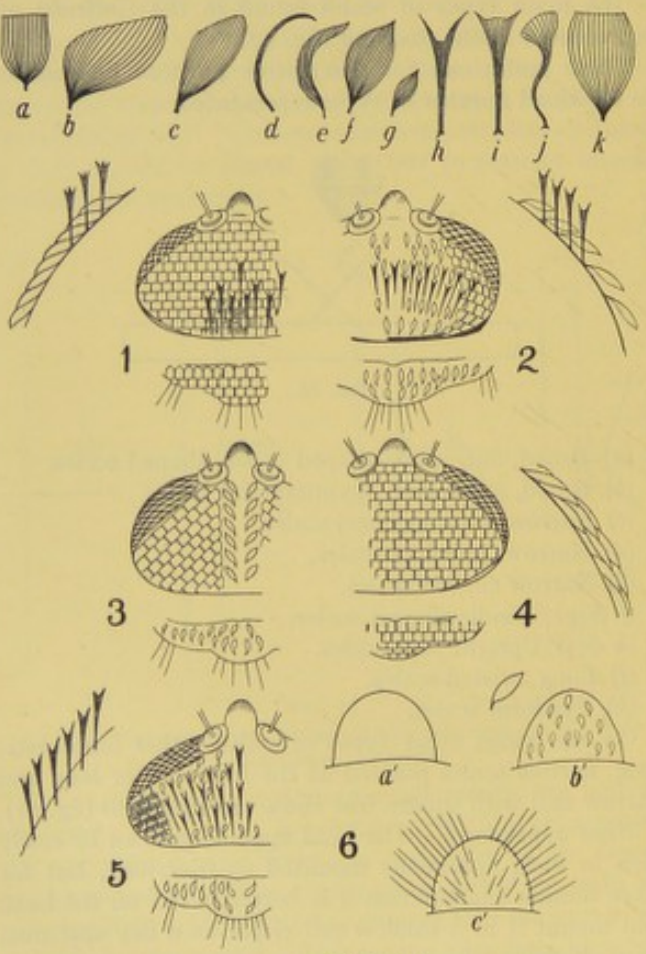


FIG. 87 (Theobald).

Types of scales, *a* to *k*; head ornamentation, 1 to 5; forms of clippers, *b*.

but not satisfactorily, for the examination of the scales, hairs, &c., as they become too transparent.

*Proboscis.**—To examine the component parts of the proboscis it is better not to use the shallow cell but to forcibly compress the head so as to cause the various component parts of the proboscis to separate; in one or more specimens all the elements can be seen.

Palpi.—The points to be noted in the palpi are their length relative to the proboscis, the number of joints, and the colour, shape and arrangement of scales and hairs. To determine the number of joints it is necessary to remove the scales off the palpi.

The antennæ.—Their length, and the relative lengths of the different joints. The number, length and arrangement of hairs and the presence or absence of scales.

The different regions of the mosquito are shown in the diagram (fig. 88). To the head are attached the appendages already mentioned, and, in addition to these, the back part of the head, or the occiput, requires close examination.

The thorax is composed of three segments fused together. The greater part is formed by the second segment, or *mesothorax*. Anteriorly on each side are two rounded projections, the *prothoracic lobes*, the remnants of the anterior segment. The posterior edge of the mesothorax is a narrow overhanging trilobed plate —the *scutellum*. The scales on this part of the thorax are of generic value.

Partly overlapped by the scutellum is a rounded mass connecting the thorax and abdomen, and is known as the *metathorax* or *metanotum*. This is the third segment of the thorax. On each side of the metathorax are the halteres.

The abdomen is segmented and has no lateral appendages. The last segment terminates in the external

* The variations in these elements of the proboscis are of no generic value.

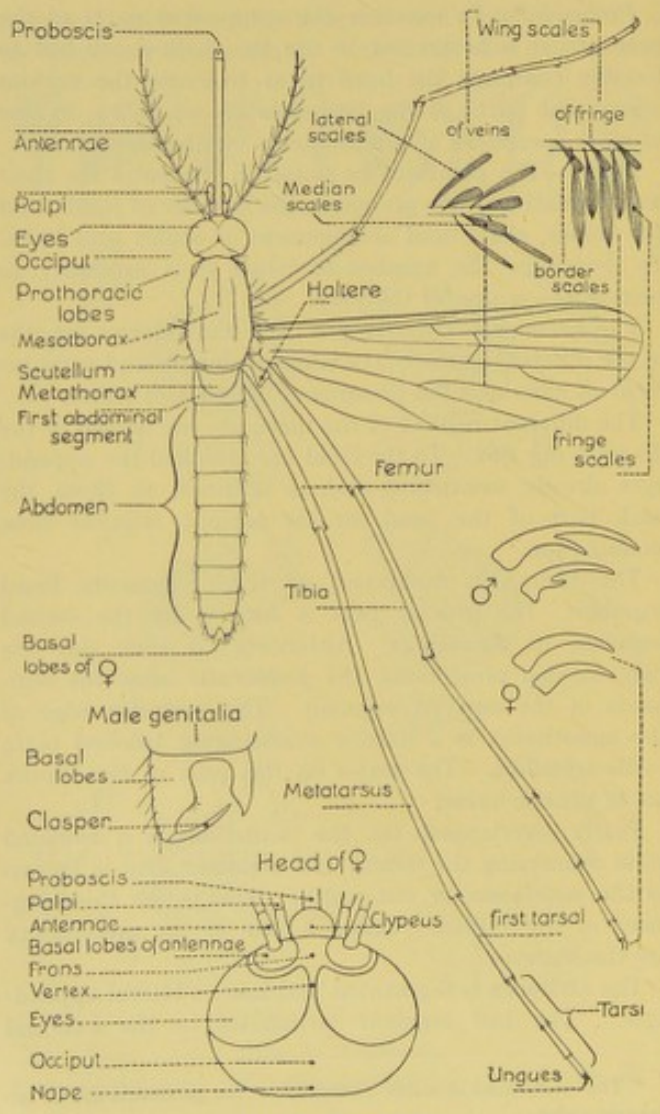


FIG. 88. (Theobald).

genitalia. These are of specific but rarely of generic value.

Thorax and abdomen.—In the examination of the dry

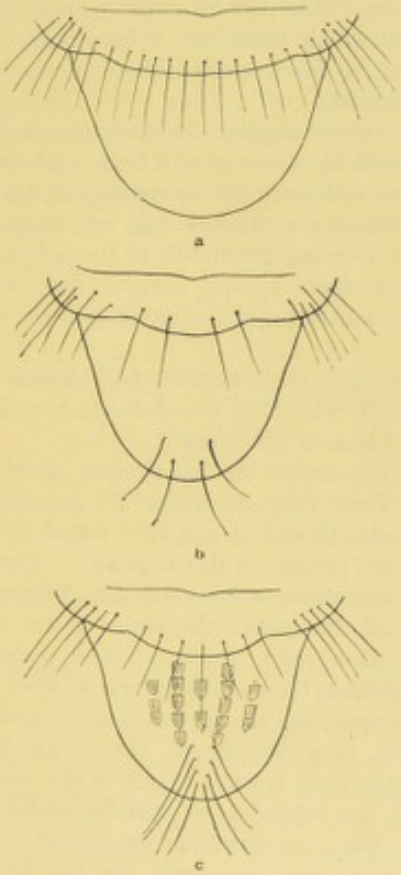


FIG. 89.—TYPES OF METATHORAX (Theobald).
a, Culex; *b*, Wyeomya; *c*, Joblotia.

mounted specimen by this method, each part of the mosquito should be examined in turn. By altering the angle in the manner described, the different parts represented

in the diagram can all be clearly made out and the character of the scales covering these parts investigated.

Metathorax.—It is well to first examine the metathorax or metanotum. This part is nude in all genera except *Wyeomyia*, in which there are a few hairs, in *Joblotia*, *Limatus*, and a few others, which have both hairs and scales (fig. 89).

Scutellum.—Overhanging the metathorax is the scutellum bordered by a row of stiff hairs and covered with scales. These scales are not necessarily of the same type as those covering the thorax, but are often the same as the scales covering the middle of the occiput, with the exception that there are no upright fork scales. The scales on the scutellum are of great generic importance (fig. 87).

The character of the scales on the *abdomen* and *thorax* are used by Mr. Theobald to subdivide the old genus of *Anopheles*. (Also of the wings.)

Occiput.—The scales on the occiput should next be examined. They vary according to genera. The upright fork scales (*h* and *i*) are only found in this situation, but are not present in all the genera. Their presence or absence, therefore, is of value for differentiation of genera. In all the genera the scales at the side of the occiput are tile-shaped scales (*a*), but whilst in some genera (*Megarhina*) these scales extend all over the middle line of the occiput, and are the only scales found, in others, as *Stegomyia*, they are found also with fork-scales; in others again they are not found in the middle of the occiput, but are replaced by spindle scales (*f*), either alone as in *Ædes*, or with narrow-curved and upright fork-scales as in *Culex*, *Mansonia*, *&c.* (fig. 87).

Wings.—The type of wing venation can be seen in a specimen mounted described as above, but is better seen in the wings when detached, flattened out and examined dry. The character of the scales covering the

longitudinal veins on the wings must be observed as these are of generic importance (fig. 91).

The wing venation of the *Culicidæ* is comparatively simple. Unfortunately no two entomologists quite agree in the names given to the different veins. Here we follow closely in this, as in other respects, the description by Mr. Theobald. It is an easy one to work with (fig. 90).

The thickened edge is called the costa, it forms the free edge of the wing. The scales on it are of no generic value; these may differ greatly from those on the longitudinal veins. The scales on the costa in all genera are

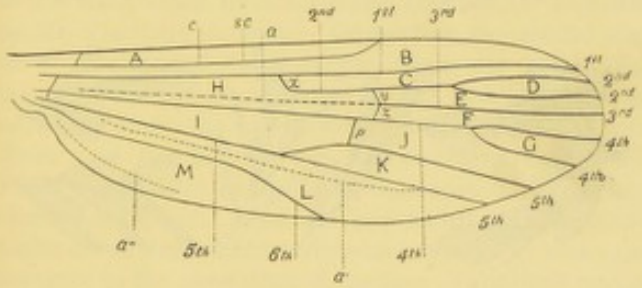


FIG. 90.—NEURATION OF WING (Theobald).

mainly lanceolate. They are of unequal length, arranged in two tiers with, at their bases, a third row arranged obliquely; these last are more like the scales on the longitudinal veins. The straight edge of the wing, with the wing expanded, is the anterior edge and is therefore so described. Next to the costa is a vein running from the base or attachment of the wing to rather more than half-way to the tip, terminating in the costa; this is called the sub-costal vein (*sc*).

The other veins running from the base towards the tip are known by numbers, the most anterior being the *first longitudinal*. This is a single vein running the

whole length of the wing and terminating at the tip. It is covered with scales in its whole extent.

The *second longitudinal* arises from the first nearly half-way from the base, and bifurcates before reaching

Fig. 91.—Various Forms of Wing-Scales (Theobald).

the tip. The space enclosed in the bifurcation (D) is known as the first fork-cell.

The *third longitudinal* arises in the base of the wing but is not covered with scales for nearly the first two-

thirds, and therefore appears nearly as a yellowish line. It does not bifurcate.

The *fourth longitudinal* arises from the base, is covered with scales in its whole extent, and bifurcates near the tip forming the second fork-cell (G).

The *fifth longitudinal* arises at the base, is covered with scales in its whole extent, and bifurcates half-way up the wing enclosing the third fork-cell (K).

The *sixth* does not bifurcate, and terminates in the costa about the middle of the posterior border of the wing. There are markings or thickenings on the wing between the fifth and sixth longitudinal and posterior to the sixth which have no scales and are not regarded by Mr. Theobald as veins. Connecting the second and third longitudinal veins is the transverse vein. The third and fourth are connected by the middle transverse ve.n, and from the fourth longitudinal to the anterior division of the fifth is the posterior transverse vein. These are definite bands of considerable thickness and often contain air. They are not scaled. The relative positions of these three transverse veins is of some importance in the separation of species. Variations cannot be relied on implicitly for this purpose, as in some species the arrangement of the transverse veins varies considerably in different individuals.

In a work of this nature it is not necessary to consider more than the identification of the commoner and more important genera into which the *Culicidæ* are divided. For full details the reader is referred to the systematic works on the subject, particularly Mr. Theobald's "Monograph on the Culicidæ," and Colonel Giles' book on "Mosquitoes."

The ordinary methods of examination have been considered, and the following synoptic table will enable the reader to differentiate the more important genera.

All the *Culicidæ* can be distinguished by the arrange-

ment of scales on the head, body and veins of the wing. Other insects which are often mistaken for them have either no scales on the wings or have the scales replaced by hairs. In any case of doubt the arrangement of the wing venation will settle the question.

There are two main subdivisions of the *Culicidæ* based on the character of the mouth-parts.

(1) *Corethrina*, in which the proboscis is not formed for piercing. These are easily distinguished from the true mosquitoes by the short proboscis. Probably they will not be long included in the *Culicidæ*.

We are concerned here with the other great division (*Culicina*). In this, in addition to the wing venation and arrangement of scales on the head, body and wings already described, the proboscis is both a piercing and suctorial organ.

In this division the characters of the *palpi* enable a subdivision to be made.

Palpi.—In one group of genera the palpi are long in both sexes; the palpi in another group are short in both sexes; and in the third and largest group the palpi are short in the female and long in the male. In each of these groups of genera further subdivisions are made on account of other characteristics, those of the scales being the most important (figs. 87 and 91).

It is far more common to find female mosquitoes than males, and to get both it is often necessary to breed the mosquitoes from the eggs or larvæ. It is therefore of practical importance to be able to distinguish the genera by the examination of females only.

The following table will show how the genus of a single female, belonging to any of the common or more important genera, may be determined by the examination of that insect. The characters of the wing and the presence of a proboscis formed for penetration will show that it is a true mosquito, and the non-plumose character of the antennæ that it is a female. If the antennæ are

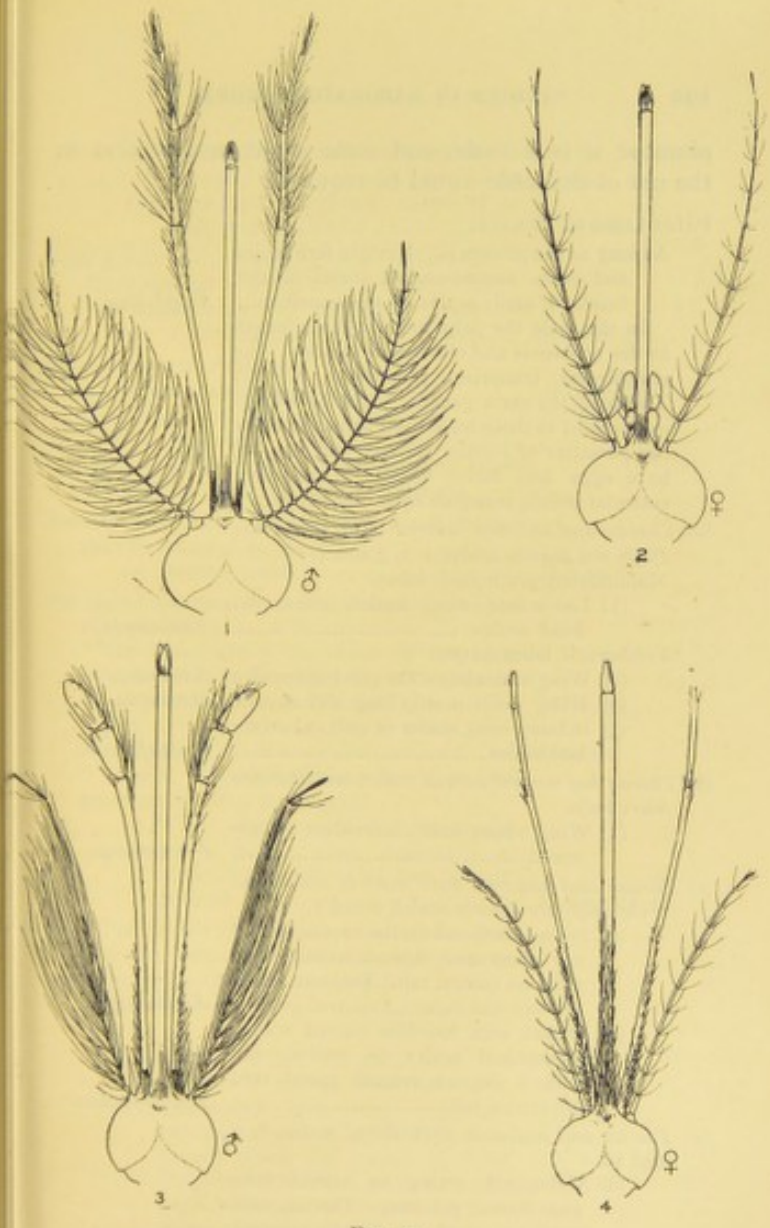


FIG. 92.

(1) Culex male; (2) Culex female; (3) Anopheles male; (4) Anopheles female.

plumose it is a male, and some slight modification in the use of the table would be required.

PALPI LONG IN FEMALE.

As long as the proboscis. Upright fork scales and often narrow-curved scales on the head. Usually scales on the scutellum... *Anophelina.*

In the male the palpi are the same length as the proboscis and clubbed at the tip. The *Anophelina* comprise, according to Mr. Theobald, ten main genera. Species belonging to most of these have been shown to carry the parasites of human malaria, and they all have eggs and larvæ which, in the more essential points, resemble each other closely.

(*a*) *Thorax and abdomen covered with hairs only. Palpi not densely scaled*, 1, 2, 3 and 4.

Mammilated prothoracic lobes.

(1) Lanceolate wing scales, some flat head scales ..... *Stethomyia.*

Prothoracic lobes simple.

(2) Wing scales lanceolate, no head scales *Anopheles.*

(3) *Wing scales* mostly long and narrow *Myzomyia.*

(4) Inflated wing scales as well as lanceolate scales..... *Cycloleppteron.*

(*b*) *Thorax has narrow curved scales, and abdomen hairs only.*

(5) Wing scales small, lanceolate or narrowed..... *Pyretophorus.*

(*c*) *Thorax and abdomen have ventral scales, and the palpi are densely scaled*, 6 and 7.

(6) Scales are found on the venter only of the abdomen. Apical lateral scale tufts, no ventral tuft. Thoracic scales are hair-like ..... *Arribalzagia.*

(7) Thorax with hair-like curved scales. Abdominal scales on venter only with a distinct ventral apical tuft. No lateral tufts ..... *Myzorhynchus.*

(*d*) *Thorax and abdomen with dorsal scales*, 8, 9 and 10.

(8) Abdominal scales as lateral tufts and dorsal patches. Thorax with narrow-curved or spindle-shaped scales..... *Nyssorhynchus.*

(9) Abdomen nearly completely scaled with irregular scales and with lateral tufts *Cellia.*

(10) Abdomen completely scaled as in *Culex* with flat scales ..... *Aldrichia.*

Palpi five-jointed, not as long as the proboscis.

Head covered with tile-shaped scales alone.

Tile-shaped scales on scutellum. Proboscis bent. First fork cell very small ..... *Megarhinus.*

PALPI SHORT IN FEMALE.

Metathorax (metanotum) with hairs ..... *Wyeomyia.*

Metathorax with hairs and a few scales ..... *Joblotia.*

In the remaining genera the metanotum is nude, and therefore we must examine other parts for further differentiation of genera.

Antennæ very long. Second joint much longer than the succeeding ones.

(a) Densely scaled ..... *Deinocerites.*

The second antennal joint is not markedly longer than the others.

The characters of the scales on the wing veins enables a further separation of the genera.

(1) Wing scales broad and asymmetrical *Mansonia.*

(2) Wing scales inflated or pyriform, symmetrical and often parti-coloured ..... *Mucidus.*

Twisted upright scales are found in this genus on the head and thorax.

(3) Wings clothed with thick elongated scales ending either diagonally or convexly, or more or less bluntly pointed ..... *Tæniorhynchus.*

The scales on the wings are narrow and their free end is squared, not lanceolate, in the other genera to be considered.

The scales on the legs aid in a further subdivision.

Legs densely scaled ..... *Psorophora.*

Legs uniformly clothed with flat scales. Subdivided into very important genera, many of which include species which are known to carry diseases. The scales on the occiput, nape of neck, mesothorax and scutellum are the diagnostic points.

No Upright Fork Scales on Head..... *Ædes and Uranotænia.*

In all these the palpi are short in the male as well as in the female. In *Hæmagogus* the palpi are five-jointed. The specimens of this genus show brilliant metallic colours.

Ædes. Palpi four-jointed. There are a small number of spindle scales in the middle of the occiput and spindle scales on the scutellum.

Uranotænia. Proboscis swollen at tip. Palpi two-jointed. First fork-cell minute.

Upright Fork Scales on Head.

Stegomyia, Culex, &c. In these genera the palpi are long in the males.

No spindle scales on occiput and tile-shaped scales on the scutellum ..... *Stegomyia and Armigeres.*

The *Armigeres* differ from *Stegomyia* in that the tarsi and abdomen are not banded. The palpi are pointed and there are no hair tufts on the male palpi.

In addition to upright fork scales, narrow-curved scales in the middle of the occiput and on the scutellum, *Culex.* This genus will probably require further subdivision ..... *Culex.**

This synopsis should enable the reader to distinguish the chief genera common in any part of the world. For distinction of species, size, colouring, and particularly the markings on the legs, thorax and wings, and slight modifications in the arrangement of the cross-veins of the wings, become important. With the *Anophelina* the markings on the posterior pair of legs are in some instances sufficient for the identification of species.

The reader is warned not to be alarmed at the apparent magnitude of the subject. It is true that this table does not give all the genera, but on the other hand, many of the genera are of limited distribution, and in few

* Many new genera have now been formed out of *Culex* and *Ædes*, vide "Mono. Culicidæ." Vol. iii. Theobald.

places will there be more than some thirty species of mosquitoes, which can readily be subdivided into their respective genera; and often the species can be easily identified by reference to the standard books, if not they should be forwarded to England for identification. The amateur, for the distinction of species, will often be much helped by examination of the eggs or larvæ, as these sometimes show more obvious differences than the adults.

For full details systematic works on the *Culicidæ* should be consulted.

Mosquitoes can be mounted in Canada balsam, but this renders the scales too transparent, and the non-scaled veins, particularly the cross-veins, are difficult to make out.

To mount in Canada balsam the insect should be placed on its back with the legs separated and the wings spread out. A small drop of thick Canada balsam is placed on a slide. The slide is then held in the hand so that the drop of Canada balsam is on the under surface and this is gently pressed against the thorax of the mosquito, and the mosquito adheres to it on lifting it. The slide should then be turned over so that the mosquito rests on it. The wings and legs should be arranged to taste and a drop of more fluid Canada balsam placed on the mosquito: as this flows over the mosquito it will cause the head appendages to spread out. A mosquito so arranged will keep indefinitely; but to complete the process a glass ring should be cemented round the mosquito, and when firmly set the cell so formed should be filled with balsam and a cover-glass placed over.

Mounted in glycerine jelly the mosquito retains its natural colouring. The best method of mounting in glycerine jelly is to make a deep cell with a glass ring and slide. Lay the mosquito at the bottom and fill the cell with the jelly by placing in hot water. Place a

cover-glass over the jelly, taking care to avoid air bubbles. It is very difficult to arrange a mosquito in the jelly. Specimens mounted in this manner often have their legs interlaced. It is a useful method for mosquitoes which have been kept in spirit, as such mosquitoes are too brittle to stand any handling and cannot be pinned out.

Specimens so mounted should be ringed with some varnish as soon as the jelly is set—otherwise evaporation will take place and the jelly shrink, resulting in the formation of air bubbles. If this accident should happen the cover-glass must be removed from the jelly and fresh glycerine jelly added. This is easily done by dissolving off the cement and gently warming to melt the jelly. The cover-glass can then be removed and fresh jelly melted and added. The cover-glass is to be replaced and when the jelly has set cemented on.

CHAPTER IX.

Dissection of Mosquitoes.

The more important parts of a mosquito are easily dissected out. The internal anatomy of the mosquito is not very complicated.

The alimentary canal is a tube with dilatations running from the proboscis to the anus, which is terminal. The mouth is suctorial and piercing and composed of the

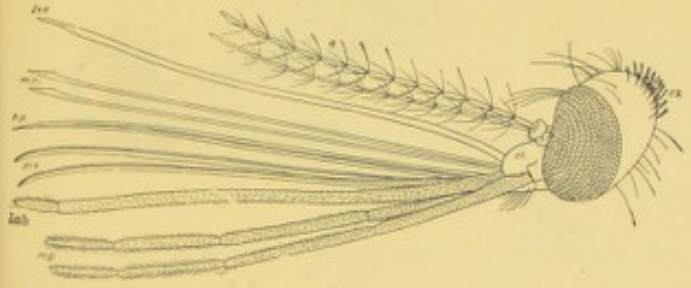


Fig. 93.

a, Antennæ; *cl*, clypeus; *lrs*, labrum epipharynx; *mn*, mandibles; *hp*, hypopharynx; *mx*, maxillæ; *la*, labium; *mp*, maxillary palps; *lab*, labella.

following parts (fig. 93). Below is a deeply-grooved, fleshy labium, this contains air tubes, is covered with scales, and forms what appears to be the proboscis, as when the mosquito is at rest the other elements are contained in the groove. It terminates in two small jointed lobes—the *labellæ*.

Above is the *labrum*, with which is fused the *pharynx* or *epipharynx*. This terminates in a sharp point. It is deeply grooved on the under surface. This groove is, by the apposition of the *hypopharynx*, converted into a tube up which the blood is sucked; this tube is continuous with the cavity of the pumping organ. The hypopharynx is a flattened chitinous rod terminating in a sharp point; it is strengthened in the middle by a ridge, and in this thickening is contained a minute tube,

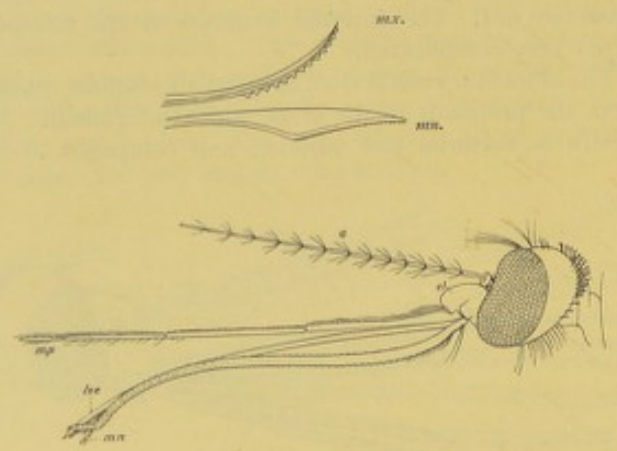


FIG. 94.

a, Antennæ; *cl*, clypeus; *mp*, maxillary palps; *lre*, labrum epipharynx; *mn*, mandibles; *mx*, maxilla.

the termination of the salivary duct. Down this minute tube the saliva is ejected into the depths of the wound made by the proboscis. There are two pairs of piercing organs, the mandibles and maxillæ; both of these are thin strips of chitin with sharp cutting edges terminating in a lancet-like point. The cutting edge may or may not be serrated. In most species the maxillæ only have a serrated edge (fig. 94).

Of these elements the labium mainly acts as a sheath and protects the more delicate parts of the proboscis from injury. It does not penetrate the skin. The tip is applied firmly to the skin and in the angle between the two labellæ all the other elements of the proboscis are thrust into the skin (fig. 95). No doubt it aids in penetration by keeping together and rendering more rigid the other elements, and as it is supplied by nerves aids in the selection of a suitable place for puncture.

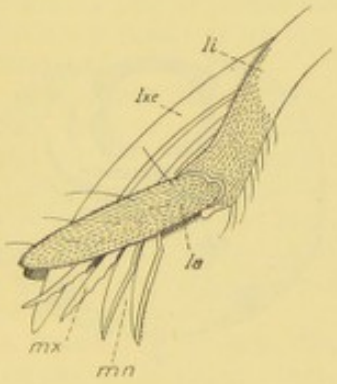


FIG. 95.

li, Labium; *la*, labella; *lxe*, labrum epipharynx; *mx*, maxillæ; *mn*, mandibles.

As the other elements penetrate the skin the labium becomes bent on itself, as depicted in the diagram.

The penetrating elements form two tubes with the mandibles and maxillæ at the sides. Up the superior tube formed by the groove of the epipharynx and the flat hypopharynx the blood is sucked, whilst the saliva is ejected through the small tube in the hypopharynx (fig. 96).

The main points in the anatomy of the proboscis

can be readily demonstrated. The hypopharynx often closely adheres to the epipharynx, so that it is the most difficult component to separate and identify.

To demonstrate the two tubes formed by the apposition of these elements, transverse sections of the proboscis are requisite. From the tubes thus formed by the epipharynx and hypopharynx the blood is conveyed into the pumping organ, which is composed of three chitinous plates, to which muscles are attached. This in turn forces the blood into a membranous tube which is con-

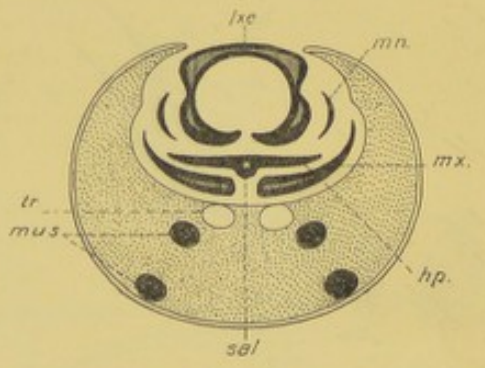


FIG. 96.

lxe, Labrum epipharynx; *mn*, mandibles; *hp*, hypopharynx: *sal*, salivary duct; *tr*, trachea; *mus*, muscle; *mx*, maxillæ.

tinuous with the commencement of the œsophagus. These parts also can only be satisfactorily demonstrated in sections.

The rest of the alimentary canal is best shown by dissections.

Mosquitoes can be killed in many ways. With those required for dissection no great precaution need be taken, as is it immaterial if the scales are knocked off. They can be killed with tobacco (cigarette) smoke, chloroform vapour, or stunned by concussion.

Dissection of Alimentary Canal.—The mosquito should be caught in a test tube. This is done by placing the test tube slowly over a resting mosquito. If it is done rapidly the mosquito will take alarm and usually escape. It is important to so approach the mosquito that no shadow falls on it. By proceeding cautiously mosquitoes are readily caught in this way, and they then fly to the closed end of the tube. With practice nine or ten mosquitoes can be caught in this manner in one tube.

Six clean slides and cover-glasses should be prepared, and on three of them a drop of normal saline should be placed; the other three should be left dry. Two sharp needles are also required.

After killing or stunning the mosquito it should be transfixed through the thorax with a mounted needle and the legs and wings pulled off and dropped on a clean dry slide. This can be easily done with the fingers, but there is no objection to the use of forceps. These can be examined dry by covering with a cover-glass and fixing this with gummed paper, or they can be mounted in glycerine jelly or Canada balsam.

The mosquito, denuded of its limbs, is placed in the saline solution on one of the slides. The posterior part of the abdomen is gently flattened with the shaft of a needle and two nicks made, one on each side, about the junction of the second and third last segments (fig. 97). This weakens the exoskeleton at that point so much that when traction is made on the last segment the exoskeleton breaks at that place.

Traction is best exercised by fixing with the point of one needle the thorax and laying the other flat on the last segment and steadily dragging away from the head.

In the space between the broken ends of the exoskeleton a series of white strands will be seen—the intestine and Malpighian tubes. On further traction the stomach and part of the œsophagus will appear (fig. 98).

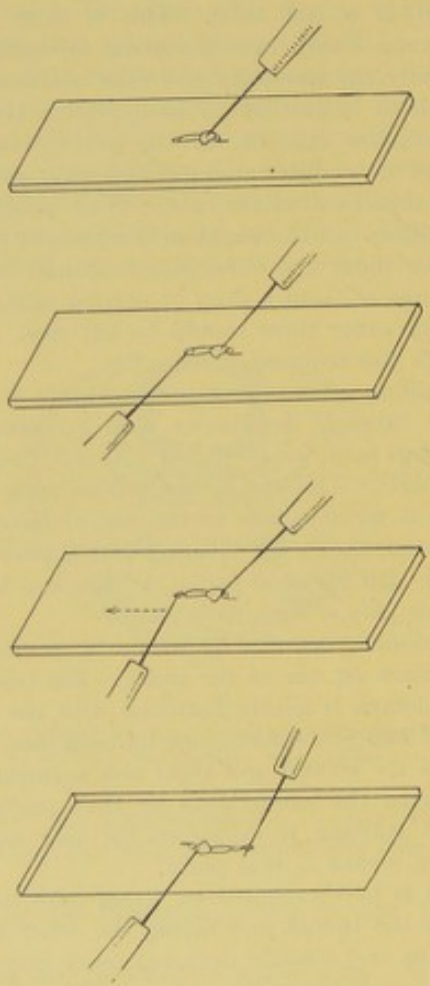


FIG. 97.

If the traction be continued from the end there is a risk that the stomach may break off. It is better to shift the needle from the posterior segments to the œsophagus at the point of emergence from the broken end of the abdomen and pull slightly obliquely on this so as to drag the rest of the œsophagus out of the abdomen and thorax. It should be covered with a cover-glass.

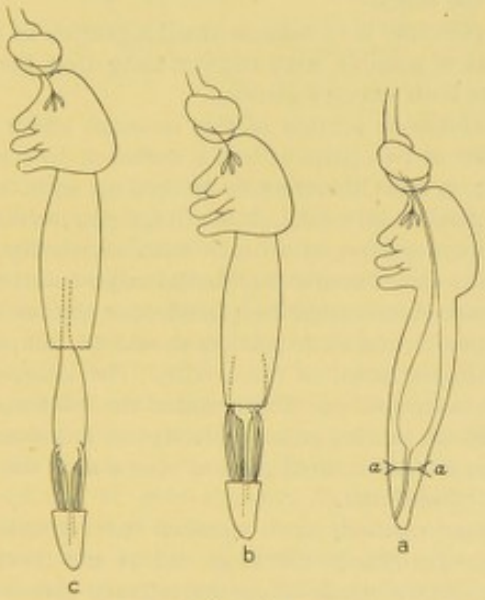


FIG. 98.

The stomach with its appendages can now be examined directly. The genital organs will be still attached to the terminal segments of the mosquito and can be examined at the same time. To show them completely it is better under the microscope to tear off the remainder of the exoskeleton of the last two segments.

The remainder of the mosquito should be placed in the drop of saline solution on one of the other slides for the dissection of the salivary glands.

To dissect the salivary glands there are several methods. The one described here is that which is most readily learnt and by which uniform results can be obtained fairly readily. It has the disadvantage that other tissues are present in the dissection and may conceal more or less of the lobes.

The principle is to take as small a portion of the mosquito as is possible with the certainty that the portion contains both salivary glands.

The chitinous portion of this remnant which includes the bases of two pairs of legs is sufficient to conceal the glands; it must therefore be broken up with the points of the needles into four or five fragments, which should be about a quarter of an inch from each other. These fragments are of course still in the saline solution.

A cover-glass should be placed over the whole series of fragments and each portion should be compressed in turn with the point of the needle. The salivary glands will be squeezed out from under the portions of the exoskeleton in the great majority of instances. It is common to find a small portion of one lobe still covered by the exoskeleton.

Another method is to squeeze the contents of the thorax out towards the head end of the thorax, after cutting off the head, when the salivary glands may be shot out uninjured. This method is uncertain, some of the lobes are often damaged, but when successful the glands are sometimes better displayed and have less surrounding tissues than the other method (fig. 99).

The first method recommended here has the very decided disadvantage that the salivary glands are not isolated but surrounded by other tissues. For permanent preparations it is not a good method as other

tissues are present. The glands do not dry quickly and become fixed to the slide as isolated glands do.

For mere examination it is satisfactory, but when confidence has been acquired by this method, if permanent preparations are desired the salivary glands must be isolated.

FIG. 99.

In the best method for isolation of the glands the head is not cut off, but the back of the thorax is separated by a longitudinal incision. A sharp edge, such as is provided by a surgical needle or cataract knife, is better than an ordinary needle. A second incision at right angles to the first is made at the level of the second pair of legs. The head is now transfixed as near the neck as possible with one needle and the remnant of the thorax fixed with another. On pulling on the head the salivary glands will be pulled out of their bed in the thorax and can be seen attached to the head. Microscopic examination under a low power objective is necessary at this stage. A final cut will separate the head, and the salivary glands are left isolated (fig. 100). It is not uncommon to find that the ends of some of the lobes have been left behind in the thorax or the glands otherwise damaged, but perfect specimens can be obtained in this way.

The excess of salt solution should be removed with blotting paper, the specimen air-dried, fixed in alcohol, and stained on the slide.

To show the relations of these parts and other struc-

tures in the mosquito serial sections are requisite. The mosquito can be cut imbedded either in celloidin or in paraffin wax.

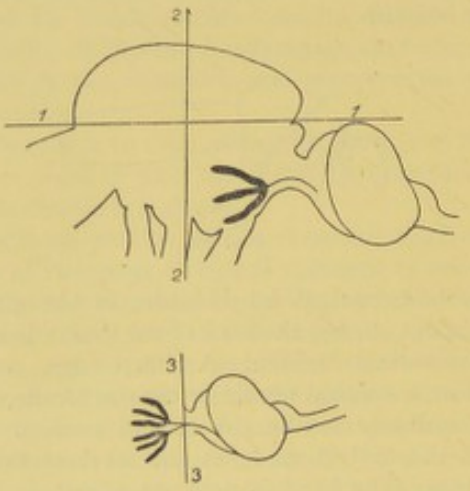


FIG. 100.

To show structure, young mosquitoes which have only been hatched for a few hours are best, and they should be placed alive in spirit and hardened in absolute alcohol.

With older mosquitoes it is better to puncture the thorax and abdomen with the point of a fine sharp knife or needle, so as to facilitate the entrance of the paraffin or celloidin.

For sections to show the development of filaria celloidin should be used, as if paraffin be used, when the sections are cut the embryos may drop out.

These methods were fully considered when dealing with methods of cutting sections (Chapter II.).

The points to be observed in the dissections are as follows :—

At the commencement of the œsophagus are three diverticula of which one is much larger than the others. These diverticula usually contain air and sometimes food. They vary greatly in size. They are often pulled out of the thorax with the œsophagus, but to show them satisfactorily it is necessary to tear off the back of the thorax and break through the upper segments of the abdomen before exercising traction on the œsophagus.

Bacteria in large numbers are found in these diverticula.

The stomach is seen as a clear translucent expansion of the œsophagus. The cells lining the intestine appear to be polygonal, and the outlines can be clearly made out by using central light only.

From the lower end just before the junction with the hind gut are seen the five Malpighian tubes, which are much more opaque and are lined by large nucleated cells, and these cells contain granules or droplets of refracting oily nature. The lumen of these diverticula is difficult to make out.

The continuation of the intestine is a tube which is not straight—the hind gut or rectum. The cells vary in different parts of the tube and the variation differs in different species. Parasites of various kinds may be found. Examination with a high power, one-twelfth inch, is necessary, as the youngest forms of the malaria parasite cannot be readily seen with lower powers, and therefore familiarity with the normal appearance of the cells of the mosquito's stomach with this power is essential.

Some confusion may be caused by the air tubes which ramify over the surface of the stomach. These appear to be black when seen by transmitted light on account of the air they contain, but silvery white when seen by reflected light for the same reason. They can be

recognised by the spiral thickening and their repeated branching.

The cells seen in the stomach form the epithelial lining of that organ. They are detached by pressure on the stomach. By making a nick in the side of the stomach and alternately floating up the cover-glass with water and abstracting it on the other side of the cover-glass with blotting paper, the detached epithelium can be removed. By repeating this process several times the epithelial lining can not only be detached but in great part washed away. This measure may be required to wash out the contents of the stomach, particularly when they are dark and opaque with altered blood. It is also necessary for satisfactory staining of malaria parasites in the wall of the stomach.

When the epithelium is washed out the stomach is reduced to a clear, transparent bag. Longitudinal and transverse markings are often seen in this and are indications of the muscular bands.

In a stomach with the epithelium thus removed the developed malaria parasites can be stained by running the stains under the cover-glass. Picrocarmine gives fair results. When sufficiently stained the excess of stain can be washed out in the same manner, and finally Farrant's solution run in to displace the water.

The stomach with the epithelium intact can also be stained in this manner, but more uniform staining is obtained by removing the cover-glass and allowing the stomach to dry on the slide. It can then be fixed in alcohol and stained with any basic stain, and after washing, dehydrated in alcohol, cleared with xylol, and mounted in Canada balsam. By this method the developed malarial parasites are not well shown, as they will not stand drying or dehydration without great distortion.

The salivary glands can be mounted in the same way,

but in the Farrant's solution the cells wrinkle and poor results are obtained. Somewhat better results are obtained by removing extraneous tissues under the microscope and drying the slide in the air. The salivary glands can then be fixed, stained and mounted. In the fresh preparation the cells will be found to vary greatly, and they are often distended with refractile droplets. These may be so numerous as to fill the cells or some of them. The cells in the middle lobe are smaller and often differ in appearance from those in the lateral lobes. The main duct has cubical epithelium, which is continued for some distance down the lobules. In *Anopheles* the ends of the ducts in the lobules are dilated, whilst in most of the genera the ducts maintain the same calibre in their entire length. Occasionally a diverticulum is met with. This may be terminal, so that the lobule bifurcates at the end, or it may be found in any other part. In *Psorophora* each gland has five lobes.

At first there may be difficulty in finding these glands with a low power. The point to search for is the main duct and its trifurcation, as this is most readily seen even if the gland is embedded in muscular or other tissues. To see the character of the glandular cells in detail an oil immersion one-twelfth must be used, and the diaphragm nearly closed as the cells are very transparent.

Sporozoa have been described in the ovaries of the mosquito, and we know that the pyrosoma of cattle is transmitted by an infected tick to its offspring.

In the present state of our knowledge it is therefore advisable to study the internal genital organs of the mosquito to some extent. These are usually removed with the stomach, but in part are hidden by the exoskeleton of the last two or three segments of the mosquito which remain still attached to the stomach. This exoskeleton can be teased off with a pair of needles;

this can be done under a dissecting or other microscope with greater certainty of not at the same time injuring the genital organs.

The female genital organs consist of a pair of ovaries opening into a common tube by the ovarian tubes. Into this common tube opens a mucous gland and also the *spermathecæ* by a long narrow duct. The spermathecæ are chitinous sacs and store up the spermatozoa received from the male. In this way by a single act of coitus by the male sufficient spermatozoa are stored up to enable many series of eggs to be fertilised.

The male genital organs consist of two testicles joined by vasa deferentia to the ejaculatory duct formed by their union. Just before this junction each vas deferens is connected by a short tube with a sac-like receptacle —the vesicula seminalis.

The ejaculatory duct leads to a short fleshy penis situated between two internal claspers, internal gonapophyses, and on each side of these are the large conspicuous external claspers.

The spermatozoa are rounded bodies with a flagellum. According to Giles they do not reach their full development in the male but in the spermathecæ of the female.

CHAPTER X.

Demonstration of Development of Malaria Parasites in Mosquitoes.

In the freshly-shed blood we saw that forms of the parasites of malaria occurred which flagellated, and that forms differing little from these did not. However much the blood was altered by exposure to air, water, or in the mosquito's stomach, a proportion of non-flagellating bodies was always present. Both those that flagellate and those that do not are the gamete or sexual forms of the parasite. They are only easily recognised in autumno-æstival fever (sub-tertian), where they appear as the crescent bodies. It is simpler to follow the development in that species of parasite on this account, though the same changes occur in the other species of malaria.

In the shed blood the crescents rapidly undergo changes if the blood be exposed to the air or moisture be added to it. If, on the other hand, air and moisture be excluded no change occurs in the crescents till they die and break up. To exclude the air a drop of vaseline is placed on the finger tip and the finger is pricked through it so that a drop of blood exudes into the centre of the oil. The oil and the contained drop of blood are transferred to a slide and the whole compressed under a cover-glass. The blood can be watched indefinitely and no change will be found to occur in the crescents till they disintegrate.

If, however, a drop of blood is taken up on a cover-

glass and exposed freely to air for two minutes and then placed on a slide, flagellating forms will rapidly appear and the crescents which do not flagellate—the females —will become round.

Instead of freely exposing to air admixture with water leads to the same result. This can be conveniently done by breathing on the slide before placing the cover and drop of blood on it.

In short, a change in the environment of the sexual forms of the parasites which does not kill them leads to transformations due to their becoming sexually active. The same changes take place in the stomach of the mosquito with greater certainty and rapidity.

To demonstrate satisfactorily these changes it is necessary to have a fairly good crescent infection. As has been already seen, the flagella from the males are actively motile, and these are the sexually active agents which enter the female and fertilise it. The process is called conjugation, and the result is an actively motile body, the travelling vermicule. This travelling vermicule contains the pigment of the female crescent and is pointed at one end.

In the stomach of a suitable mosquito—several species of *Anopheles* for human parasite and *Culex fatigans* for proteosoma—the vermicule passes out of the stomach cavity and becomes encysted in the stomach wall, forming the *zygote*. About thirty-six hours after feeding on an infected person these encysted zygotes will be found and can be readily recognised by their pigment, which at this stage can be seen to be little changed from the pigment of the parasites from which they were derived. They are best seen in fresh specimens, but can be stained with any basic stain and seen after the epithelium has been removed from the stomach. The youngest forms are a little larger than a red blood corpuscle, but they rapidly increase in size, though at a rate varying with

the temperature of the air. At about 80° F. they attain their full development in *Anopheles funestus* in twelve days.

With this increase in size there is, of course, no increase in the pigment, as the zygote does not derive its nutriment from the blood. The pigment therefore is relatively scanty and absorption or solution of it must take place, as it frequently disappears completely.

When fully grown the zygotes attain the size of 50 or 60 μ. The growth of the parasites is entirely outwards into the body cavity of the mosquito and away from the *lumen* of the intestinal tube, so that when mature they appear to be globular excrescences stuck on to the stomach.

The contents of the zygote first divide into a series of segments called *blastophores*. These blastophores soon lose their smooth outline and have an irregular, shaggy appearance, which as they become more mature is seen to be due to the conversion of the outer part of the blastophore into a mass of filaments attached by one end to a small central residual mass. When quite mature these filaments break off and the cyst is then filled with these filaments, which are narrow bodies pointed at both ends and about 14 μ in length. These bodies are known as zygotoblasts, blasts, sporozoites, or exotospores.

In the fresh specimens they can be seen only in specimens immersed in saline solution or in weak 1 per cent. formalin solution.

To observe them the fresh dissected stomach in one of these solutions is covered with a cover-glass, and by gently moving this cover-glass with a needle the stomach can usually be rolled over a little so that one of the mature zygotes is seen in profile projecting from the edge of the stomach (fig. 101). Pressure with a needle on the cover-glass will now cause the rupture of the capsule

of the zygotes and the contents, the blasts or sporozoites will be poured into the surrounding saline solution and can then be examined. If quite mature the contents will be entirely composed of sporozoites with a few small round masses of residual protoplasm, and in some cases a few small grains of pigment that have escaped absorption.

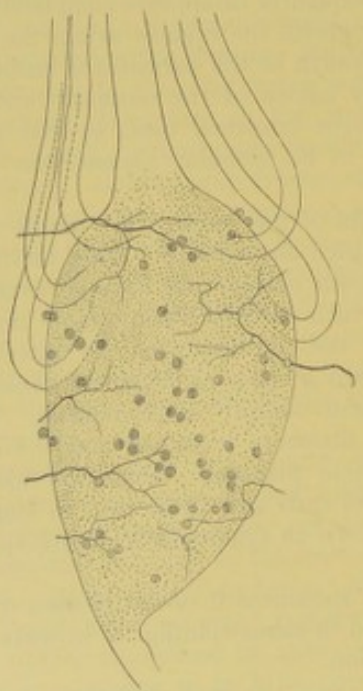


FIG. 101.

If not quite mature some of the sporozoites will remain attached to the protoplasmic residue which formed the centre of the blastophore, forming a tangled mass round this centre.

If empty cysts are found attached to the stomach detached sporozoites will be found in the fluids from any part of the body of the mosquito, and in some of the cells in the salivary glands they will be found in large numbers.

Even with a low power the invaded cells in the salivary gland can usually be detected, as they present a granular appearance, and with a high power oil immersion the individual sporozoites can be made out unless they are too numerous. In such a case by pressure on the cover-glass the cells may be ruptured and sporozoites will be poured out in a manner similar to that in which they were poured out on rupture of a mature zygote.

The cells in the middle lobe of the salivary glands are the ones which most frequently contain sporozoites, and usually cells in the middle lobes of both glands are invaded, but they may be found in cells in any of the lobes.

The demonstration of the development of *Filaria nocturna* in mosquitoes is even simpler.

In the first twenty-four hours the filariæ will be found living in the stomach and will be seen to be actively locomotive and to have cast their sheaths (*ecdysis*). Empty sheaths may also be found. Later the filariæ will be found after the removal of the stomach by teasing out the muscular masses, especially those of the thorax. Normal saline solution should be used as pure water is apt to destroy the worms.

By dissecting daily one or two of a number of mosquitoes found to carry this filaria and fed at night on a person harbouring *Filaria nocturna*, every stage in the development can be traced.

Filaria nocturna has been shown to be carried by several species of mosquitoes belonging to several genera; *Culex fatigans*, *Panoplites Africanus*, *Anopheles argyrotarsis*, and *Anopheles costalis* are amongst these.

So far experiments with *Stegomyia* always fail because, though occasionally the filariæ make their way into the muscles and become encysted, development is slow and incomplete and the embryos die and become absorbed. *Anopheles funestus* does not carry *Filaria nocturna*.

Temperature has an important influence, and at low temperature, even with a suitable species of mosquito, no development takes place, and at intermediate temperatures development is much retarded.

The points to observe are the alterations in size and shape, the variations in mobility, and the formation of intestinal and other structures.

The embryo as found at first in the muscles is exactly like the embryo freshly escaped from the sheath. It soon becomes less actively motile and thicker. The extreme tail of the worm does not become thicker, so that we soon have a body like an elongated sausage with a small thin tail. This tail retains its mobility longer than any other part of the worm. The embryo increases in length, an alimentary canal with a terminal mouth and subterminal anus is formed and the mobile tail disappears. At this stage only very sluggish occasional movements can be observed. The embryo continues to elongate and again becomes actively motile. At this stage the alimentary canal is complete and there are three small projections developed at the tip of the tail.

The actively motile young filariæ now escape from the muscles and passes towards the head of the mosquito and from there into the labium, where they can be found stretched out with their heads towards the tip of that organ.

It will be remembered that the labium is the only part of the proboscis that does not penetrate the skin.

For the young filariæ to obtain access to man it is therefore necessary that they must escape from the labium and find their own way down the puncture made by the other elements of the proboscis.

The most probable supposition as to the course taken is that the worms make their escape through the thin membrane stretched between the bases of the labella. This membrane is the weakest part of the labium and is put on the stretch when the two labella are pushed against the skin and separated as the piercing elements of the proboscis are plunged into the skin. In the angle between the two diverging labellæ the young worms would readily burst through this membrane and enter the hole made by the other parts of the proboscis, or they might be pushed into the wound as they escaped with the piercing elements of the proboscis. That the filariæ escape from the labium in this way was surmised by Drs. Annett and Dutton, and has been shown to be the case by Bancroft.

The further development of the young filariæ in man is not known. At the last stage of development in the mosquito the worms are not only small but sexually immature.

Further growth and impregnation of the female must take place in man before embryos are again formed, appear in the blood, and are in turn taken up by mosquitoes.

It is obvious from the above that a patient harbouring adult filariæ with embryos in his blood can not only cause infection of others but continued and repeated reinfection of himself.

Another filaria known to be carried by mosquitoes is *Filaria immitis* of dogs. This is a sheathless filarial embryo, but instead of passing through the walls of the stomach of the mosquito it passes up the lumen of the Malpighian tubes and there it further develops and passes through its non-motile stage. When the larvæ again become motile they burst through the Malpighian tubes and work their way through the tissues of the mosquito to the head and enter the proboscis just as the young *Filaria Bancrofti* does.

The demonstration of the development of the filaria is best done with fresh specimens of infected mosquitoes by teasing them out on a slide in normal saline solution. Even the demonstration of the young worm in the proboscis can be done by breaking across the proboscis, and the various changes in the motility of the embryo and young worm can only be demonstrated in this manner.

Sections in celloidin are the best for permanent specimens, as the worms can then be seen in their proper position.

Nothing is known of the mode of development of the other human filariæ. Experiments with many species of mosquitoes have failed. Other blood-sucking insects may be the carriers or intermediate hosts, as is known to be the case with filariæ of some of the lower animals.

A large number of species of filariæ have been described in birds. The intermediate hosts are unknown, and much information as to the possible methods of the propagation of filaria might be obtained by systematic experiments on some of these birds.

Various protozoa, such as gregarines and sporozoa, have been found in mosquitoes or in their larvæ.

Bacilli swarm in the intestinal tubes of mosquitoes; they are particularly abundant in the air sacs or diverticula from the upper end of the œsophagus. Of diseases usually assumed to be bacterial, yellow fever has been shown to be carried by mosquitoes (*Stegomyia fasciata*). The organism of this disease is not known, and though there is reason to believe that some development of the unknown organism takes place in the mosquito, nothing is known of the changes that take place.

Dengue Fever.—It is stated that this disease is carried by mosquitoes.

CHAPTER XI.

The eggs of mosquitoes of different genera vary greatly. In most cases they are laid on the surface of water. A few species of mosquitoes will lay eggs in other situations. Some of these, whilst in captivity, can be induced to lay on many damp surfaces—wet blotting paper, the cut surface of apples, potatoes and the like. *Culex dorsalis* and some at least of the *Stegomyia* lay eggs in this manner.

Most of the species belonging to the restricted genus *Culex* lay their eggs in masses or rafts. Each individual egg has its long axis vertical or nearly so to the surface of the water, and as the lower end is slightly the larger the mass formed by the aggregation of these eggs rests with a convex surface downwards on the water and a concave surface upwards. The egg masses when first laid are white but soon darken, usually to a black or dark brown colour, but in some species to a bright bronze. The individual eggs vary according to species. In all the upper end is plain, but the lower may be plain, spiked or ornamented with a whorl. There is no definite operculum that can be seen in the unopened egg, but when the larva bursts through, the egg shell ruptures in a circular manner round the broad end of the egg, and the lid thus formed is pushed aside by the larva. When the eggs hatch the raft to a large extent breaks up.

In *Anopheles* the eggs are quite different. They are never laid in rafts but deposited in little groups on the surface of the water. After a time when disturbed by

superficial currents in the water or in the air they become scattered and arranged in patterns which vary according to the nature and proximity of the sides of the vessel, or to floating bodies such as blades of grass, pieces of stick, &c. The eggs lie horizontally on the surface of the water and are irregularly spindle-shaped with the upper surface flattened. They are covered with a thin reticulated membrane which is closely adherent to the upper and under surfaces, but thrown into loose folds at the sides so as to form a projecting ridge running a distance varying according to species towards both the pointed ends. This fold is strengthened by transverse thickenings and air is contained between the folds. The *Anopheles*' egg, therefore, has on each side an air chamber or float attached which prevents the egg from sinking. If the egg does sink, or if, when it has become adherent to the sides of a vessel, it is submerged it does not hatch, nor does it if once thoroughly dried. When the larva hatches the egg-shell splits obliquely towards the thicker end, and pushing aside the cap thus formed the larva makes its escape.

The eggs of *Stegomyia* are also laid separately. They are oval eggs and are covered completely with a reticulated membrane. No large air cells are present, but at first there is air in some of the small reticular spaces. The eggs may remain floating and hatch, but more frequently sink and hatch after remaining some hours or even days submerged. *Culex dorsalis* lays similar eggs, and such eggs are more resistant than those of most mosquitoes.

The species of *Panoplites* most frequently observed rarely lays eggs in captivity. The eggs are oval and projecting from one end have a long tube terminating in a slightly expanded, trumpet-shaped opening.

The eggs of *Psorophora* are not unlike those of *Stegomyia* in shape but are rather more pointed. According to Dr. W. N. Berkeley they are "prickly."

Eggs are best obtained by collecting adult female mosquitoes and keeping them in a small cylindrical vessel, a wide-necked 4-oz. bottle is suitable, containing water. It is well to have some twigs or fragments of grass floating on the water for the mosquitoes to rest on. The top of the vessel should be covered with mosquito netting to allow air to have free access. They should be fed on blood as often as they will feed.

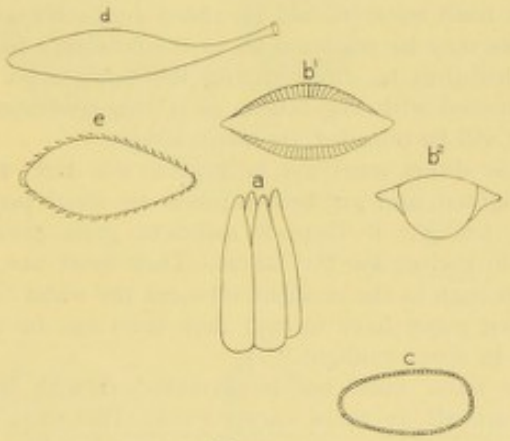


FIG. 102.

a, Eggs of *Culex*; b^1 b^2, Eggs of *Anopheles*; *c*, Egg of *Stegomyia*; *d*, Egg of *Panoplites*; *e*, Egg of *Psorophora*.

Larvæ can be obtained by keeping the eggs in water at a suitable temperature. When first hatched they are small and quite white, but they soon increase in size, and either in part or as a whole change colour.

They are voracious and require abundance of food, but with many species of mosquitoes, particularly with some of the *Anopheles*, the water must not be putrid or peaty.

A white, flat dish, such as a half-plate or full-plate

photographic tray, is as good a breeding place as any. Some earth should be placed at the bottom, and it is well to place some grass with the roots and earth attached in two or three places, both along the edge and also towards the middle, so as to form at least one islet. The dish should be filled so that there is about three-quarters of an inch depth of water, and these dishes are best prepared a few days before the larvæ are placed in them. A little of the "green slime" or other algæ found growing in fresh water should be added and a few grains of dry rice may be scattered about the bottom. Abundant food will thus be supplied, but the water must not be overstocked with vegetation, as if this decomposes the water will be unsuited for many larvæ.

These dishes must not be kept in the dark, must be well lighted, and are best exposed for short periods to direct sunlight if there is sufficient grass growing to provide shelter for the larvæ. They must not be left long enough in the sunlight to warm the water.

When pupæ have formed they must not be exposed at all to direct sunlight.

The water must not be overstocked with larvæ as they are all at times carnivorous. The larvæ should all be about the same age, but may be of different species. Some large larvæ will destroy the young of both their own and other species.

The tops of the dishes should be covered to prevent the entrance of dust, a plate of glass, or better, a larger glass dish inverted over the dish, will suffice.

Larvæ can either be raised from the eggs or caught from natural waters by scooping up the water in any receptacle. Where large numbers of larvæ are required any receptacle from a bucket downwards will do, but where larvæ are scanty they are best caught by using a dipper. An ordinary white enamelled coffee-cup serves the purpose well, but in some situations a longer handle

is better and this can be fitted on to the cup, or a soup ladle may be used.

Some larvæ are most numerous at the edges of pools or streams in the shady places. In using the dipper the open mouth should be turned towards the bank and plunged in inclined so that the water from the edge

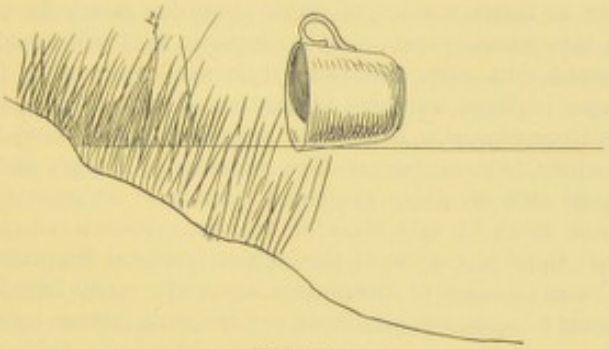


Fig. 103.

Fig. 104.

rushes in. The dipper should, as soon as the rush of water has ceased, be turned upright and removed from the pool. It should be allowed to stand for a few minutes till the mud has settled and then examined. A hand-lens is useful as the very young larvæ can easily be overlooked. (Figs. 103 and 104.)

In obtaining the specimen care must be taken not to disturb the water in any way before using the dipper, as larvæ readily take the alarm and dart to the bottom.

In shallow pools and small puddles larvæ can be readily seen by looking rather obliquely at the undisturbed surface of the water. When they occur in such situations they are usually numerous. In running water and in larger masses of water they can rarely be seen in this manner, and unless a dipper is used will be overlooked. In such situations it is not common to find them in large numbers in any small surface of water, and consequently the dipper may have to be used frequently to demonstrate their presence. Though in any small area of water examined they may be scanty the total areas of this class of breeding place is so great that these places are of the highest practical importance.

Some species of mosquitoes are more easily found as larvæ because the adults do not frequent human habitations. During certain seasons, particularly cold and dry seasons, larvæ of all species will be found more readily than adults.

In making collections of mosquitoes it is well both to breed from adults collected in as many different classes of place, houses, cattle-sheds, grass and forest, as possible, and also to rear adults from larvæ or eggs found in still and running waters, natural pools, small and large, and also in artificial collections of water.

No water, even that in cess-pits, is too foul for some species, whilst others will not breed in water that Europeans consider fit to drink.

The pupæ are found in the same situations as the larvæ; they appear as small black objects which are usually motionless unless disturbed.

The larvæ and pupæ can be transferred from the dipper to a wide-mouthed bottle for carriage. Both larvæ and pupæ are easily destroyed if the water is kept

in motion, as they do not rest on the surface sufficiently long for proper respiration. If it is necessary to carry them for long distances it is well to make frequent halts every half hour to an hour and place the bottle containing the larvæ upright in a shady place for a quarter of an hour or so.

Anatomy of Larva and Pupa.—The larva is divided into three regions—the head, thorax and abdomen. The abdomen is segmented and there are nine segments. The larvæ vary in colour in different species, but even in the same species variations occur according to the degree of exposure to light and the nature of the food. In the more transparent larvæ the colour of the intestinal contents, green or brown, is more obvious than that of the larva itself.

The head is a rounded mass joined to the thorax by a narrow neck. It is covered with chitinous plates to a large extent, particularly on the dorsal surface. There are a pair of compound eyes and also a pair of simple eyes. The appendages are the antennæ, mandibles and maxillæ, and there are also numerous hairs or bristles.

The thorax has attached on each side bunches of bristles which probably serve as balancers.

The abdomen. The anterior segments at the posterior edges of the lateral surface carry similar bristles. The eighth segment is marked by the termination of the respiratory tubes either on the surface or in chitinous tubular projections jointed on to the upper surface of this segment. This projection is known as the respiratory syphon. The ninth and last segment is smaller and more cylindrical, and at the posterior end the intestine terminates at the anus; surrounding it are four long papillæ each containing a branching trachea. These papillæ are in movement in life and may be much retracted. There are long hairs usually in tufts arranged on the ninth segment. The arrangement of these varies

considerably in different species, and the description of any larva should contain an account of the arrangement of these hairs.

The alimentary system of the larva consists of a tube, apparently structureless and of uniform calibre, running from the mouth to the anus. In the more fully-grown specimens this tube is seen to be contained inside the intestine arranged as in the adult. In the space between is clear fluid not containing any food particles. The Malpighian tubes and other appendages of the alimentary canal of the adult are present at this stage.

The intestinal system including the inner tube containing the food and the outer tube and appendages can be pulled out of the larval case in a manner similar to that by which the intestine is removed from the adult, but it is more easily done by extraction through the anterior part of the larva than from the tail.

One species of gregarine passes the stage of encystment in larval mosquitoes. These gregarines pass up the Malpighian tubes of the larva and there become encysted. The cysts rupture during the pupal stage and the young gregarines pass into the alimentary canal of the pupa or young imago, and are passed out with the first excrement voided by the imago.

The space between the temporary and permanent intestine also contains as well as these gregarines numerous micro-organisms, and the possibility that organisms of certain species may be taken up by larvæ and develop or be conveyed by adults is one of the many points in connection with insects that might repay investigation.

The respiratory system of the larva is comparatively simple. At the end of the respiratory syphon, if there be one, or from the dorsal surface of the eighth abdominal segment, are the openings leading into the two main tracheæ, which pass up the abdomen giving off branches to each segment and inosculating freely in the thorax.

They send off branches to the various parts here and to the head. The character of the commencement of the respiratory tubes is of great importance in the determination of genera and species.

The duration of the larval stage varies greatly, scarcity of food and low temperature both retard the development.

The length of life possible depends on the species. The larvæ of some mosquitoes can survive the whole of the English winter, though very little development takes place. The larvæ of other species under circumstances unfavourable for development do not keep alive for more than a few weeks.

The larvæ of mosquitoes which are able to keep alive under circumstances such as cold unfavourable for their development are said to hibernate as larvæ.

The Pupa.—When the larva has reached its full stage of development the thorax becomes swollen, casts its cuticle with all the appendages, and becomes a pupa. The organs are already formed.

The pupa differs most materially from the larva in that there is no longer a mouth opening externally, and the respiration is conducted through two tubular openings arising on each side of the compound head and thorax. The change in appearance is great, the head and thorax are fused and the only external appendages are the two respiratory tubes. The abdomen is still segmented and is usually curved, so that the termination is under the compound thorax. It terminates in two large fins.

The pupal stage is a comparatively short one. There is no possibility of feeding and the pupa remains quiet, breathing through the respiratory tubes unless disturbed, whilst the more complete development of the imago takes place within its sheath. The duration of the pupal stage is affected by the temperature, but is usually from two to five days. The pupa of some species will not

remain alive longer than a few days if the conditions are not favourable for development.

In the examination of eggs, larvæ and pupæ, the points to be observed are as follows :—

Eggs.—(1) The size, shape, colour. (2) The manner in which the eggs are arranged and where deposited. (3) The character of any thickenings or other external markings. (4) The length of time required under stated conditions, temperature and so on, between the deposition of the eggs and the hatching of the larvæ, and any variations noted with variations of conditions. (5) The effect of desiccation, immersion and temperature on the vitality of the eggs.

Larva.—In the larva the relative sizes and shapes of the different divisions—head, thorax and abdomen. The character of the head appendages, the antennæ, mouth apparatus, &c. Any marked colouring. Much work has been done on the differences in the appendages of the head of *Anopheles* larvæ, and it has been shown that the differences are so marked in their arrangement that many of the species can be distinguished as larvæ.

In the thorax the character of the lateral hairs and any characteristic markings must be noted.

In the abdomen the points of greatest importance are the appendages on the eighth and ninth segments. The presence or absence of a respiratory syphon attached to the eighth segment is one of the most important generic differences. Where present it varies in length and shape in different genera. In different species it varies in colour and in the distribution of colour so markedly that it is often easier to distinguish between different species by the character of the syphon than it is to distinguish between the adults. With the differences in length or to the absence of the spyhon are associated varying positions of larvæ.

The arrangement of bristles and hairs on the eighth

and ninth segments present marked differences in the different species. In *Anopheles* on the other segments, in addition to the lateral hairs there is on each side a row of stellate or palmate hairs. These are nearer the middle line than the simple bristles, and the stellate portion forms a kind of cup. This adheres to the surface film of the water and aids the larva in maintaining its horizontal position.

Colouring of larvæ is of less importance, as in some species the colour may vary from yellow to green, brown, or even black. In others variations are comparatively small, these are usually dark under all circumstances.

In noting the colour any conspicuous markings must be mentioned, the conditions under which the larva was grown, and whether or not change of conditions such as greater light, different food, &c., results in a change of colour.

The nature of the food can be determined by the examination of the contents of the intestine or by watching the larvæ feed in water containing a mixture of natural foods. It will be found to vary. The kind of food on which they thrive best should be noted.

The duration of the larval stage under as many diverse conditions as possible, including exposure to light, heat, and cold, and any observations as to the conditions predisposing to death or leading to an undue proportion of males in the imagines, should be noted.

The natural enemies of the mosquito larvæ, fish, larvæ of other insects, &c., are of great importance. Where possible the species should be determined. If the larvæ are caught as larvæ and not reared from eggs particular care should be taken to observe the nature of the places in which the larvæ were found.

Breeding Places.—It is convenient to divide these into permanent waters such as will withstand a considerable period of rainless weather, and *temporary* waters

which require frequent renewal. They may be natural or artificial. Of permanent waters, rivers, large ponds and the edges of lakes under certain conditions are of the utmost importance. In such situations the larvæ are usually widely scattered, and without the repeated routine use of a dipper such places, often the most important, are usually overlooked.

The conditions favourable are the growth of grasses, reeds or sedges in the water. These growths check the stream, provide food, and protect to some extent the larvæ from their natural enemies.

There are two main classes of growths important :—

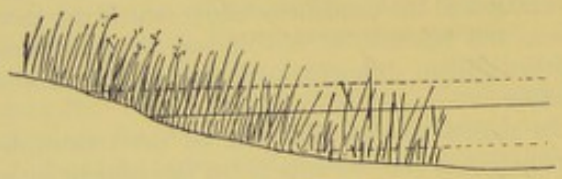


Fig. 105.

(1) Those growing from the bed of the river or lake, in the shallows and on shelving banks. The height of the water greatly affects the area suitable. The taller and thicker sedges are not so suitable as the lower and thinner ones, probably on account of the absence of light and too great stagnation of the water (fig. 105).

(2) Those growing from floating masses of roots and attached to the earth only near the edge of the river. The raft formed by the closely-interlaced roots is submerged by the weight of the grass growing in the air, and in the shallow water lying above this raft of roots *Anopheles* larvæ breed freely. No alteration in the level of the water makes any material difference to this, a common class of breeding place (fig. 106). In flood times islets of this floating grass are torn off and carried down

the stream, carrying with them larvæ, and in this manner they may be carried long distances down the river. It is not improbable that the cutting of the sudd in the Nile may result in larvæ of mosquitoes at present unknown in Egypt being introduced there.

Rivers are dangerous when variations in level are not too great or too rapid. Such streams as have a constant supply independent directly of the rainfall are particularly dangerous. Such sources are the melting of the snow from snow-covered mountains and the effluents of large lakes.

Fig. 106.

Springs which often arise on the slopes of hills are other important permanent breeding places. These usually commence as a small pool with a surrounding swampy area. The grasses round are often of different species or grow more luxuriantly than elsewhere, and these places can therefore usually be identified with ease.

The streams arising from such springs are not of much importance during heavy rains, but when the water supply is diminished, wherever the streams spread into swampy areas, or form pools fringed with vegetation, or in back waters, larvæ are usually to be found with the aid of the dipper. In some of these situations they are carried by the stream from the springs or other breeding places. In others the eggs may be deposited and hatch in the place in which the larvæ are found. Amongst the easiest places to find larvæ are the pools left in the bed of such a stream when the spring com-

mences to dry up, particularly if a small current connects the pools and keeps the water fresh. Some springs will dry up after a month's dry weather, others in three or four months, but some are usually permanent though the water may be scanty from one wet season to another.

Swamps unless kept supplied by fresh water are not suitable breeding places for some species. For other species they are suitable provided the vegetation is not too rank.

A high-level subsoil water may lead to formation of natural permanent pools. On the sandy shores of great lakes the sand is usually thrown up into a ridge with a hollow behind it, and in this hollow as long as the lake level is high water will be present and forms a suitable breeding place. As the lake level reaches its greatest height at the end of the wet season and very slowly falls in the dry season, these pools may persist in the vicinity of such lakes for some months after the rains have ceased.

Temporary Breeding Places are of many different classes. Almost any hollow or hole that will contain water is a suitable breeding place during continuous rains. If the rain be intermittent only such places as can retain water during the periods of intermission are suitable. Such places require a frequent and heavy rainfall and an impervious soil, and are not often found except under these conditions. The "*Anopheles*' pool" most often described belongs to this somewhat exceptional class.

A place that is frequently flushed is not a suitable breeding ground, but irrigation trenches or natural hollows are good breeding places if the area of the trenches is such that the water supplying it is insufficient to flush it in its whole extent.

Artificial Breeding Places.—Borrow pits at the sides of railway embankments, the trenches so often made

in the course of road-making, and hollows or furrows made in native and other gardens, are common breeding places of *Anopheles* and some other mosquitoes. A high level of the subsoil water is necessary for these places to be of importance.

Irrigation systems where the water supply is continuous but insufficient to flush are important places. In any case, even with a well-designed system, if the source of the water be from a natural breeding place larvæ will be conveyed all over the irrigation system.

Instances occur in which larvæ are conveyed for over a mile by such a trench from a natural permanent breeding place to a European settlement.

Obstructions in the course of a stream, such as Irish crossings, dams, &c., may convert an inferior natural breeding ground into an excellent one.

Broken bottles, water butts, empty tins and any artificial receptacle that will hold water are preferential breeding places for some species of mosquitoes, particularly those belonging to the genus *Stegomyia*.

Wells in many places do not seem to be breeding places, but in other places they certainly are.

On the whole, artificial breeding places are usually the work of Europeans, and the worker in the Tropics has rarely to go beyond his own grounds to find larvæ of several species of mosquitoes.

Too little attention has been paid to the breeding places of different species. We know that great differences occur in the preferential 'breeding places of different species as of different genera, but little exact work has been done on the subject.

For exact descriptions of the larva of one species which might serve as an example the reader is referred to the articles on *Anopheles maculipennis*, by Nuttall and others, in the first volume of the *Journal of Hygiene*.

The Pupæ differ less from each other than the larvæ,

and many insects form pupæ that are not unlike those of the *Culicidæ*. The greatest differences are to be observed in the respiratory tubes. In all the *Culicidæ* they are simple tubes with one opening. In the *Anopheles* the opening of the tube is a wide, expanded, trumpet-shaped one; in the *Culex*, *Stegomyia*, &c., the opening is more of a slit and the termination is little extended. In *Panoplites*, according to Dr. Low, the tubes are very long and slightly bent forward. In the different species there are variations in the size of the pupa and in the colour. The majority are, after exposure to light, brown or black, though when first formed they are yellow. A few are green, though most of these become dark before maturity.

To hatch the pupæ all that is required is that they should not be disturbed and that they should be kept in clean water. No food is needed. They should be kept in a half light.

Carriage of Mosquitoes.—Mosquitoes may be carried in any stage of their existence. As eggs they are not very easy to carry, as those that float are often washed on to the sides of the vessel and there are dried and killed. Eggs like *Stegomyia*, which sink and are not injured by immersion, are easily carried, and it is probably owing to this that this mosquito or its larvæ are so often found on board ship.

The carriage of larvæ we have already dealt with. For the development of many light is a necessity, and consequently such species, including many, if not all, the *Anopheles*, that require light are not carried far by sea, as most of the fresh water is necessarily in closed casks or other dark receptacles.

The adult mosquitoes must be carefully carried as they are easily injured by rough handling or bruising. On the whole glass vessels should be avoided because of the hard surface of the glass. Mosquitoes cannot

hold on to it. If grass or other substances be placed in the glass vessel water of condensation is often deposited on the glass and the mosquitoes adhere by the wings to this wet surface and speedily die.

If glass vessels, test tubes, &c., are used, the mosquitoes must be carried very carefully, and on no account must water be placed in the vessel or grass or other substance containing moisture.

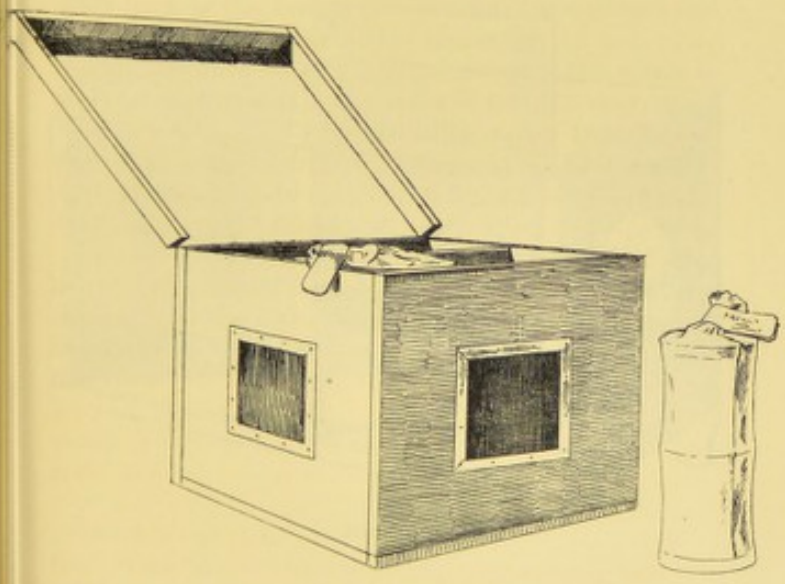


FIG. 107.

A light cage covered with mosquito netting is as good an arrangement as any, though at a pinch a small box covered with netting on the open side will work satisfactorily.

The box designed by Dr. Sambon and containing four compartments. each containing a cylindrical wire cage

covered with netting, is an excellent one (fig. 107). It was in such cages that infected mosquitoes were sent from Italy to the London School of Tropical Medicine for the well-known infection experiments, which resulted in the practical demonstration that mosquitoes infected with the malaria parasite could infect men in a country where there was no other possibility of acquiring an infection.

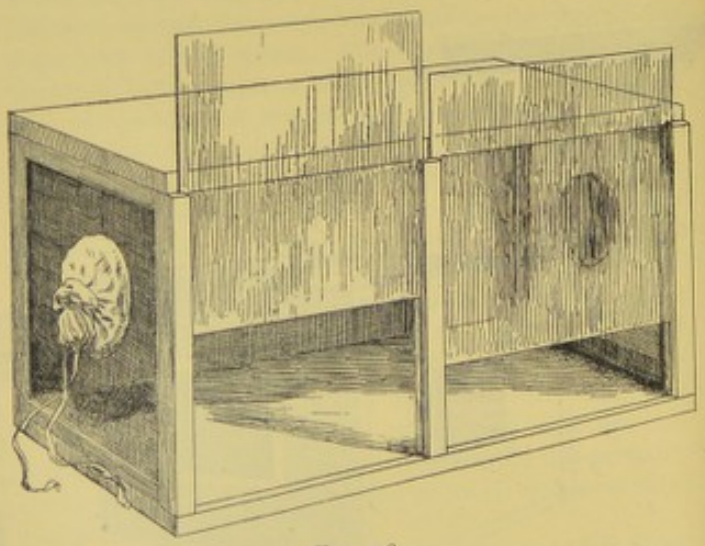


FIG. 108.

Adult mosquitoes can be kept in test tubes or wide-necked bottles covered with fine gauze or mosquito netting. A little water should be placed at the bottom and some resting place, such as a piece of stick, twig of grass or folded card placed above the water.

Many mosquitoes will feed readily through the netting, others will not though they feed readily in a larger space.

Mosquitoes thrive better if kept in a larger space.

The box slightly modified from Dr. Sambon's cage is very convenient for this purpose. The front is composed of glass in two pieces for convenience in packing, whilst the ends are of fine wire gauze to allow the entrance of air. The hole in the centre of this gauze is covered by cotton sleeves. These are convenient for the introduction of an arm for feeding experiments. Through these holes the hand and test tube can be introduced when we wish to catch a mosquito. The pieces of glass forming the face of the box slide in a groove and can be removed when required (fig. 108).

Ripe fruit, such as apples, dates and bananas, serves as food for mosquitoes, but some will not lay eggs unless supplied with blood. As substitutes for fruit, sugar, syrups or jams will serve. Some species, *Stegomyia* for example, are easy to keep in captivity and can be kept alive for months; others will die in a few days.

Ants of many kinds are very destructive to mosquitoes, particularly when these are confined in small spaces. To avoid this the mosquito cage should be placed on legs, each of which rests in a small tin containing kerosine.

CHAPTER XII.

Pigment Deposits and Degenerations in Tissues.

The pigment deposits are in the main those derived from altered hæmoglobin. Of these the most important is melanin, the residue from the digestion of the red corpuscles by the parasites of malaria. This pigment is taken up by the leucocytes and other phagocytic cells and deposited in various parts of the body.

As seen in the interior of a parasite it is usually finely divided and varies in colour according to the species of parasite. In the large masses which are found in the tissues it is black with a slight greenish tinge. It is insoluble in acids, in alcohol and in ether, but is readily dissolved by alkalies. It is very stable and is not destroyed by putrefaction. In solution in alkalies it has a distinctly greenish tinge, but shows no characteristic bands when examined with the spectroscope. From this solution it can be precipitated by the addition of acids, and by repeated solution in alkaline fluids and precipitation with acids, can be isolated in an impure condition. Analysis shows that it is very rich in iron, more so than hæmoglobin or any of the other hæmoglobin derivatives. The iron is in firm organic combination, and does not give the inorganic iron reactions. After frequent precipitations it becomes brown in colour and the same brownish tinge can be observed in pigment left in certain organs as a result of old malarial invasion.

Melanin in an acute attack of malaria is found (*a*)

in parasites, (*b*) in leucocytes, and (*c*) in certain cells of the connective tissue type in the liver, in the parenchymatous cells in the spleen, and occasionally in the nuclei of the endothelium of the capillaries in various parts of the body, such as the brain, liver, suprarenals, &c. In an invasion of short duration the only pigment found is in small granules, often no larger than those set free after the breaking up of a sporulating body. At this stage the cells containing the pigment stain normally and do not differ in appearance from other cells of the same type which do not contain pigment.

Where death occurs some little time after the termination of malaria the distribution of the melanin deposits is much more limited. It will not be found in the red corpuscles as there are no parasites, nor in the leucocytes or endothelial cells, but will be restricted to the connective tissue cells in the liver and the parenchyma of the spleen. The pigment itself is now aggregated into larger masses, though these may be seen to be composed of separate granules. The cells stain faintly with ordinary stains and often appear to be shrunken or distorted. If examined still later the pigment will only be found in blocks or masses, and the cells containing these blocks will not take basic stains, and appear merely as an outline round the pigment masses, some of which appear to be free. The change to a brownish colour, particularly at the edges of such masses, is sometimes to be noted.

Later, even months or years after the malarial attack, pigment may be found. If present it will be imbedded in the fibrous stroma and no trace of the cells will be seen. At this stage the spleen is usually the only organ in which the pigment will be found.

A consideration of these changes will show that the date of a malarial invasion or invasions can be inferred from the melanin deposits in the organs if the state of

division of the pigment, the staining reactions of the cells containing the pigment, and the situation of the pigment be observed.

It will not be concluded because no pigment is present that there has not been antecedent malaria. We have proof that pigment is rapidly removed from all organs but the liver and spleen (and occasionally the lymphatic glands), and also that it may be removed from either the liver or spleen. In all recent attacks both organs contain pigment; after a longer interval it may be present in the spleen only, or more rarely in the liver only.

In the older cases where malarial infections have not occurred for a prolonged period the amount of melanin found is usually small. In some cases even a few weeks after an attack of malaria the amount of melanin is so small that careful search is required to reveal it.

It seems probable that as long as the pigment is contained in living cells it is fairly readily removed.

In many cases there is evidence that the pigment (melanin) deposited is the result of several distinct attacks, as in the same specimen finely-divided pigment in cells which stain normally, coarse pigment in cells that stain poorly, blocks of pigment with no traces of a containing cell, and pigment in between strands of fibrous tissue can all be seen.

Melanin is the only pigment which is characteristic of malaria.

Another pigmentary deposit of a bright yellow colour is often found in the organs in cases of malaria, but this is also found in pernicious anæmia, in the anæmia of ankylostomiasis, and in other cases where hæmolysis or blood destruction has taken place.

This yellow pigment differs from melanin not only in colour but in that it is insoluble in alkali as well as in acid. It appears to be slightly soluble in alcohol. Whether or not it contains iron is difficult to ascertain,

as it is frequently associated with other substances containing iron in loose combination. When found alone it usually does not give the reactions for inorganic iron.

This yellow pigment is found in the hepatic cells, in the secreting cells of the kidney, particularly in the first part of the convoluted tubules, and in the spleen.

It is evidence of blood destruction from any cause, whether acute, as in blackwater fever, or chronic, as in pernicious anæmia or ankylostomiasis.

Both these pigment deposits can be observed without cutting sections by making "squash" preparations of tissues of the organs, but the arrangement is better shown in sections.

To merely detect the pigment no stain is needed, but to show the character of the cells containing the pigment it is well to stain lightly. Hæmatoxylin gives good results, but a better stain is carmine, as both the melanin and the yellow pigment stand out better against the red background. Thionin should not be used as it has an affinity for these pigments or the protoplasm surrounding them.

In many cases granules that contain iron in a condition to react to the usual tests for inorganic iron are associated with the yellow pigment. Ammonia sulphide is sometimes used as the test for the demonstration of inorganic iron, but has the disadvantage that the brown sulphide of iron deposited can be confused with malarial pigment. A better reagent is ferrocyanide of potassium in an acid solution as the blue ferrocyanide of iron is characteristic and causes no confusion.

The section should be first treated with a 2½ per cent. aqueous solution of potassium ferrocyanide for five minutes and then with a 1 per cent. solution of hydrochloric acid in glycerine. This acid glycerine should be slightly warmed, and must be left on till the blue colour is quite distinct. If the blue colour only shows faintly

the specimen can be replaced in the ferrocyanide solution and again treated with acid glycerine. The specimen can then be washed in water, dehydrated in alcohol, cleared in xylol, and mounted in balsam.

It is important that the sections should not be touched with iron after they are cut, so that they must not be lifted with a needle, as in any place touched with iron there may be a deposit of the blue ferrocyanide of iron.

Loosely combined iron will be shown blue, whilst the melanin in which the iron is in firm combination will remain black. The yellow pigment may be in part turned blue or may be unaltered. The outlines of the cells can generally be seen and counter-staining is not necessary, but weak carmine solutions can be used if it is desired.

The iron may be diffused throughout the cells or may be found in granules either alone or mixed with yellow pigment.

The relationship of these ferruginous granules to the yellow pigment is not definitely known. In the most acute hæmolytic processes, such as in blackwater fever, both are present and the iron-bearing granules are the most numerous. In the most chronic forms, such as some cases of ankylostomiasis, yellow pigment alone will be found.

The balance of evidence is in favour of the view that ferruginous granules are evidence of active and recent hæmolysis, whilst the yellow pigment is a more permanent substance and though formed as a result of acute hæmolysis is, when in considerable amount, evidence rather of a prolonged chronic hæmolysis.

Some authorities hold other views and consider that the yellow pigment when old gives the iron reaction.

The important point is that both these substances are proof of blood destruction, they are not evidence of malaria although often found in cases of malaria.

They are evidence of the general blood destruction that may be caused by the parasites of malaria as well as by other organisms.

In the vicinity of certain skin lesions there may be considerable disturbance in the normal arrangement of the pigment, so that instead of being deposited only in the deeper layers of the epidermis it is scattered not only in the superficial layers of the epidermis but also in the subcuticular connective tissues.

The disturbance of the arrangement of pigment is most conspicuous in growths of the granulomatous group, including lichen hypertrophicus.

Pigment is normally present in the skin and it is common to find pigment in the mucous membranes, particularly of the mouth in the coloured races. Such pigment is generally found in patches in the mucous membrane of the tongue, cheeks, or gums. It has no connection with malaria or other disease, but is more conspicuous in cases of advanced anæmia, as the pigmented patches then stand out more markedly against the general white background.

The normal pigmentation of the pia mater has been already alluded to.

The pigment in all these cases is much less soluble in alkaline solutions than the melanin of malaria. Pigmentation of the skin as a result of Addison's disease is also well known.

In melanotic sarcoma, black or brown pigment is also deposited in the growth.

Degeneration. — Cells exposed to various influences undergo degenerative processes. Death or necrosis of cells may take place, and in such cases the cell ceases to stain normally, so that instead of taking up basic stains it stains with acid stains, or feebly with both acid and basic stains. The nuclei break up and lose their characteristic staining reactions and the whole cell may

disintegrate and be converted into granular *débris*, or "caseation may take place in which a mass of cells is replaced by granular fatty material. This may calcify."

Where the morbid influences are insufficient to cause cellular death, changes occur in the protoplasm. Of these the more important are: (1) "Cloudy swelling," in which the protoplasm of the cell becomes swollen and the aspect of the cell changed so that its contents become obscured and very finely granular. This change is best seen in fresh, unfixed cells and is shown in stained specimens by an irregularity in the staining. This change occurs in the early stages of inflammatory action and may be general in any prolonged pyrexia.

(2) Fatty degeneration may affect any cells, but more especially muscular fibres and the glandular cells of the liver, kidney, intestinal mucosa, &c.

In this form droplets of fat are found in the interior of the cells: these at first are small, but in advanced cases the whole contents of the cell appear to be replaced by fat and the nucleus is squeezed to one side.

With fresh specimens the high refractive index of the fat renders the diagnosis easy. In specimens passed through alcohol, &c., the fat is dissolved out and the condition is then recognised by the meshwork of the protoplasm having clear, round, unstained spaces which were previously occupied by the fat globules.

Special methods show this form of degeneration more clearly. In specimens hardened in any of the osmic acid fixatives, such as Flemming's solution, or cut fresh and treated with weak osmic acid, the fat will be stained a deep and intense black. Such sections can be counterstained with safranin. Soudan No. 3 is also a good stain for fat. Fresh tissues or tissues hardened in formalin must be used. The sections are treated with a saturated alcoholic solution (80 per cent. alcohol) for fifteen minutes, rapidly washed in 50 per cent. alcohol

and washed in distilled water. They can be counter-stained with hæmatoxylin and mounted in any glycerine medium. The fat will be stained a deep red.

A rough estimate of the amount and extent of the fatty degeneration may be made from such section, but the main advantage of the sections is to show the distribution of the degeneration and the class of cells mainly involved.

A promising method for the estimation of the extent of this degenerative process is the determination of the specific gravity of the organs. In some cases the fat is in sufficient amount to cause the entire liver to float in water, but more commonly it is short of this. To determine the specific gravity a large portion of an organ is weighed and the volume of this portion determined. This volume can be ascertained in the course of an ordinary *post-mortem* examination by the use of a vessel with an open tube fixed at the side.

The vessel is filled with water till the water escapes from this tube. When the water has ceased to escape a receiver is placed under the tube and the weighed portion of the organ is placed in the vessel. Water will again escape from the tube, is collected in the receiver and measured. The volume of this water is the same as that of the organ placed in the vessel, as it is the amount displaced by it.

We now know the volume of a given weight of the organ and therefore its specific gravity. This method is sufficiently exact for ordinary purposes if a sufficiently large piece of the organ is taken, but for comparative purposes more information is required than we at present possess as to the normal variations in the specific gravities of organs.

Fatty Degeneration is an important factor in many tropical diseases. It is marked in yellow fever almost as much as in poisoning by phosphorous. In the anæmia

of ankylostomiasis it is constant and pronounced, and as it affects extensively the intestinal mucosa, it is, in the more chronic cases, largely responsible for the impairment of the digestive processes in some of these cases.

Amyloid Degeneration is best shown in fresh sections. Macroscopically it can usually be determined by treating a cut surface of an organ with tincture of iodine; a deep brown colour is produced in such portions as contain this amyloid material.

Sections can be similarly treated and mounted in glycerine media.

Methyl violet stains amyloid material a deep red, standing out clearly from the surrounding violet.

Amyloid degeneration is not common in tropical diseases, with the exception of leprosy. In that disease, even when there has been no extensive suppuration, amyloid degeneration is fairly common.

Fibrous Degeneration. — As a result of degenerative changes in many parts, and particularly in the nervous system, the nerve elements are replaced by fibrous tissues. These are well seen in spinal diseases in which degeneration of nerve tracts is followed by the formation of fibrous tissue in the tracts occupied by the degenerated nerves. This fibrous tissue stains with ordinary basic stains and is well shown by carmine. This change is sclerosis, but is the final result of the degenerative changes. The early nerve degenerations require special and complicated methods and could not be satisfactorily studied without special knowledge and appliance.

The simplest method for demonstration of the early nerve degeneration is that of Marchi. The tissue is hardened in Müller's fluid for one week. It is then transferred to mixture of Müller's fluid 2 parts and osmic acid 1 per cent., aqueous solution 1 part. It is left in this solution for one week and either hardened in formalin 4 per cent., frozen and cut, or re-hardened

in alcohol and imbedded in celloidin or paraffin. The sections must not be mounted in xylol balsam or any substances which dissolve fat. They can be mounted in glycerine.

Some of the changes can be detected in nerve filaments teased out, treated with 1 per cent. osmic acid and mounted in glycerine.

CHAPTER XIII.

Fæces.

The examination of fæces is of the greatest importance as most of the intestinal entozoa deposit their eggs whilst in the intestinal canal, and these become mixed with the fæces. In other cases the parasites themselves may be passed, or in the case of tape-worms the mature segments or proglottides may be passed.

In dysentery, cholera, &c., the organisms found in these diseases are present and can be isolated from the stools.

Microscopic examination of the stools is very necessary and much information can be gained. The stools can be examined as passed in any vessel, but are more conveniently examined if passed into transparent glass vessels; these can be covered with a larger glass cover fitting over the lower vessel like an enlarged Petrie dish.

The points to observe are:—

(1) The presence or absence of blood, mucus, muco-pus or pus, and the arrangement relative to the stool of such a discharge.

(2) The colour of the stool and its consistence.

(3) The presence or absence of evidence of gaseous fermentation.

(4) The odour.

(5) The reaction, determined as soon as possible after the stool is passed.

(6) Any visible signs of animal parasites, such as the

worms themselves or the proglottides or segments of tape-worms.

(1) Mucus alone, or streaked or mixed with the blood, indicates inflammatory action in the lower bowel, not necessarily dysenteric. It may be caused by anything that sets up such inflammation, such as bilharzia, ulcerated hæmorrhoids, chronic ulcerations of various kinds of the rectum. These include malignant growths, granulomatous growths, and the ulceration left as a sequela of dysentery.

Clear mucus, whether streaked with bright blood or not, without any admixture of fæcal matter, is met with in early or acute dysenteric attacks. Turbid or purulent mucus, sometimes in large quantities and passed either without any stool or with solid formed motions, is more indicative of a chronic ulceration, from whatever cause, of the rectum.

With ulceration limited to the rectum stools are often coated with mucus. The more intimately the mucus and blood are mixed with the fæces the higher up are the lesions from which the mucus or blood is derived. In some lesions the mucus is so intimately mixed with the fluid fæces that it is difficult to discern, but tilting the vessel from side to side will often indicate its presence by the manner in which the stool flows.

Blood may be passed, bright red or in clots, in large quantities. This is no proof that it is passed from the rectum, as if in sufficient quantity and not mixed with the fæcal contents of the intestine it need undergo very little change in passing through the large intestine. Such blood is occasionally passed in ankylostomiasis. If intimately mixed with the fæces it may have lost completely the red (blood) colour and appear black and tarry—melæna.

In other cases, though still red, it has a duller colour, more like anchovy sauce. Such stools are passed in

some cases of dysentery where the small intestines are implicated, and may also be passed in cases of extensive enteritis secondary to malaria.

Microscopic as well as macroscopic examination of the mucus and blood, as well as of the stool, should be made.

(2) The colour of the stool is much modified by the diet, milk especially causing pale stools. Articles of diet taken by a patient have a marked effect, and amongst abnormal articles that may be met with are earths of various kinds, coal-dust, &c., which to the inexperienced may cause much confusion. The alcoholic stools of jaundice may be simulated by those of some cases of earth-eating, and the black stool of the coal-dust-eater has been mistaken for melæna. The dark blue stools passed by patients taking methylene blue are easily recognised.

The consistence of the stool is of great importance, and it will be found that looseness of stools is of more importance in tropical practice than in England. In ulceration of the cæcum and upper part of the colon, even when this is acute and extensive, there need be neither visible mucus nor blood, nor even tenesmus. "Tropical diarrhœa" is frequently shown at *post-mortem* examinations to be dysenteric. It is very fatal. On the other hand, mucus and blood may be passed, and yet the stools be formed when there are a few chronic ulcers high up in the large intestine.

(3) In some forms of tropical diarrhœa, particularly that form known in the East as sprue, the stools passed are full of air-bubbles and are undergoing active gaseous fermentation.

(4) The odour varies so greatly with the diet that it is of minor importance. In the races subsisting mainly on a scanty vegetable diet the odour is singularly slight. In cases of dysentery associated with formation of sloughs the ordinary fæcal odour is replaced by the peculiar penetrating smell of the condition.

(5) The normal acid reaction of the fæces is in many cases of diarrhœa and dysentery replaced by an alkaline reaction. To determine the reaction the fæces must be examined as soon as they are passed, as a change rapidly occurs in most fæces, particularly when fluid, rendering them alkaline.

(6) Parasites of various kinds may be seen by direct examination, but more often it is necessary to strain the stools. This is best done by placing the stool on a muslin or wire gauze strainer, which should be strong,

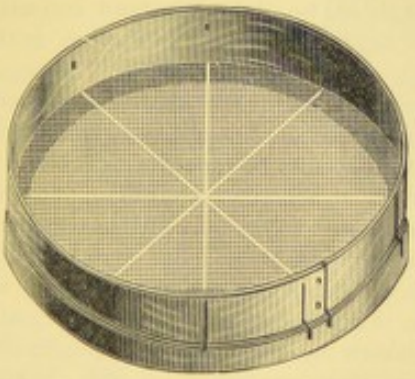


FIG. 109.—WIRE GAUZE STRAINER.

and adding water and stirring well. By repeating this process all the smaller particles of the fæces will be carried through the muslin, and only the coarser particles and any entozoa present will be left on the strainer. Some of the smaller entozoa may be carried through the strainer, but that is unusual. If this is suspected the fluids that have passed through can be strained again (fig. 109).

Microscopic Examination.—The most important objects of this are (1) the detection of ova of parasites, (2) the

detection of animal micro-parasites, and (3) the investigation of the bacteria present.

Ova are readily seen with a low power two-third inch objective, but for their identification at least half an inch or, better, quarter of an inch objectives are requisite.

The preparation of the stool is very simple. A small particle of the fæces is placed on a slide, it can be conveniently taken up with a splinter of wood such as a match stick. If not too hard it should then be compressed by a cover-glass into a thin layer; if too hard for this it can be mixed with a little water. If the stool be watery it should be allowed to stand, and with a pipette some of the fluid taken from the bottom, as the eggs are heavier than a fluid stool and sink. The eggs that may be met with are those of the *Ascaris lumbricoides*, *Trichocephalus dispar*, *Ankylostomum duodenale*, *Oxyuris vermicularis*, several species of tape-worms, several species of *Distoma*, those of *Bilharzia hæmatobia*, and the embryos of the *Anguillula intestinale*. The attached diagram shows the appearance of the more important of these ova.

The eggs of *Ascaris lumbricoides* (round worms) are enclosed in a thick, irregular capsule usually stained yellow or brown by the fæcal colouring matter. They have a thin, clear, unstained internal capsule, and the protoplasm contents are granular and do not as a rule completely fill the inner capsule (fig. 110, *a*).

If too much pressure has been used the outer capsule may have been ruptured and the egg seen surrounded only by its thin transparent capsule. Even then it can be readily distinguished from the eggs with thin capsules by its more spherical shape, and particularly by the granular and unsegmented character of the egg contents.

The eggs of *Trichocephalus dispar* (whip-worms) are easily distinguished, as they are small oval eggs contained in a thick, deeply-stained outer capsule which has an

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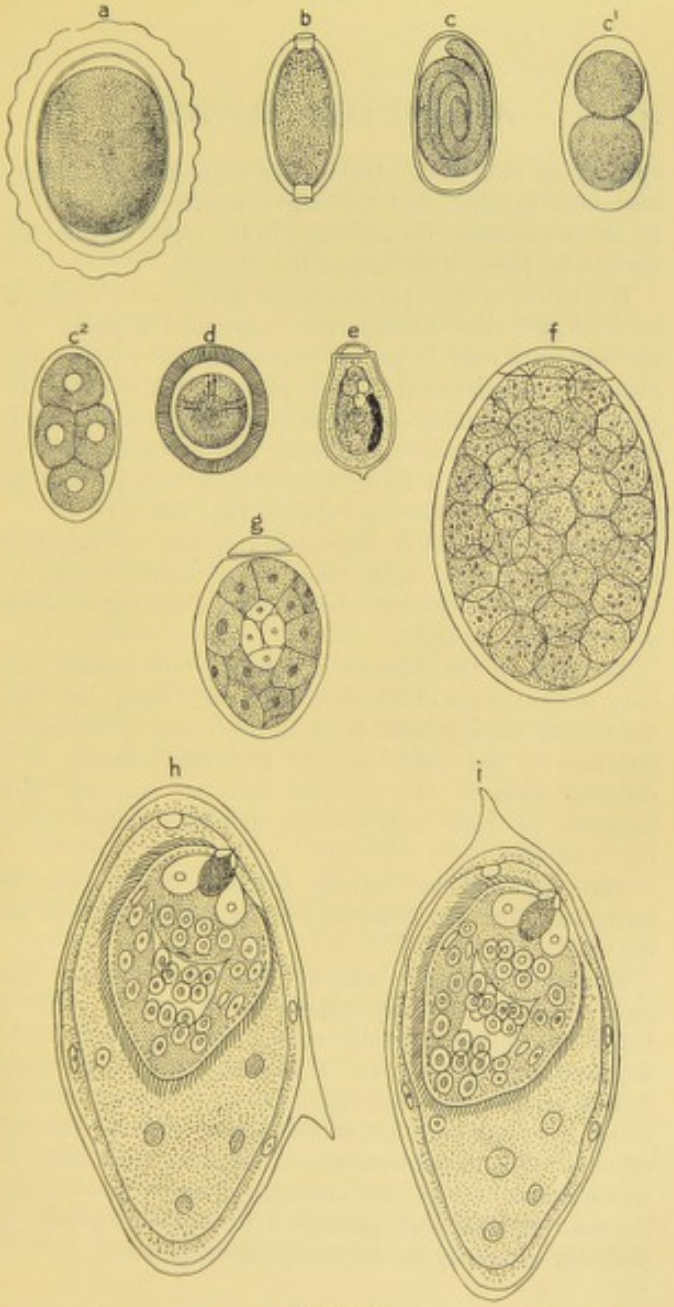

FIG. 110.
a, *Ascaris lumbricoides*; *b*, *Trichocephalus dispar*; *c*, *c*¹, *c*², *Ankylostomum duodenale*; *d*, *Cestode*; *e*, *f*, *g*, various *Distoma*; *h*, *i*, *Bilharzia*.

opening at each end. Inside this capsule is a thinner, unstained capsule, and the egg contents are granular. In many instances the openings in the outer capsule are seen to be plugged by mucus (fig. 110, *b*).

The ovum of the *Ankylostomum duodenale* is enclosed in a single, thin, transparent, unstained capsule. At the time the egg is passed segmentation usually into about four segments has taken place, but if the stool be kept a larger number of segments will be present according to the time and temperature, and in twenty-four to forty-eight hours a fairly well-formed embryo will be found in many of the egg capsules (fig. 110, *c*.)

Oxyuris vermicularis (thread-worm) has an egg that in size and general appearance is not unlike the ankylostome. At the time the stool is passed this egg contains a well-formed embryo.

The eggs of the different species of tape-worms may only present slight differences from each other, but they are readily distinguished from all other ova by the radial striation of the thick capsule and the presence of a differentiation in the contents into an embryo. The hooklets of this embryo can usually be made out (fig. 110, *d*).

The eggs of the various *Distoma* (flukes) can be recognised by the presence of an operculum or lid (fig. 110, *e*, *f*, *g*).

Bilharzia hæmatobia has a highly characteristic egg, as if armed with a sharp spike. In eggs passed with the fæces this spike is usually at one side, but it may be terminal (fig. 110, *h* and *i*). If water be added to the fæces it will be seen that the egg contains a ciliated embryo which soon becomes active and bursts through the egg capsule. The free-swimming embryo remains alive in water for some days, but undergoes little further change. An intermediate host, perhaps a fresh-water mollusc, is probably necessary for its further development.

Only one embryo is found in the stool—the embryo of the *Anguillula intestinale*. The embryos of the *Trichina spiralis* are very rarely passed in the stools as they normally penetrate the intestinal walls and pass into the surrounding tissues.

In the fæces, thread-worms, segments of tape-worms, and occasionally round-worms are passed naturally. After the administration of powerful anthelmintics the whole tape-worm, round-worms, ankylostomes, flukes, and whip-worms may be passed. Some species are never found under any circumstances in the fæces.

The worms met with in the human intestine and its appendages belong to the following orders :—

(1) *Cestodes*. These flattened worms have a segmented body, no digestive tube, and are hermaphrodite.

(2) *Trematodes*. In these the digestive tube is incomplete ; there is no anus and the body is not segmented. They are usually hermaphrodite.

(3) *Nematodes*. These have a complete digestive tube. They are cylindrical worms and they are not hermaphrodite.

The human cestodes are : *Tænia solium*, *T. saginata*, *T. confusa*, *T. Africana*, *Dipylidium caninum*, *Hymenolepis murina* (*T. nana*), *Davainea Madagascariensis*, *Bothriocephalus latus*, *Diplogonoporus grandis*.

Cestodes or Tape-worms.—The embryonic or cystic forms of the *Tænia echinococcus* may be found in the liver, muscles of man, &c. The definitive host is the dog. These cysts, the hydatid cysts, can hardly be mistaken for non-parasitic cysts ; they can be readily distinguished if there is any doubt by the laminated cyst wall and the presence of hooklets in the cyst or discharges.

In the case of the *echinococcus* man is the intermediate host. A larval non-cystic form of *Bothriocephalus* (*B. Mansoni*) has been found in the connective tissues of men in Japan, and the same larval form has been

obtained from an aboriginal of British Guiana. These larvæ are probably the larval form of a *Dibothriocephalus* or of a *Diplogonoporus*.

The greater number of the tape-worms found in man attain sexual maturity in him. Man is therefore the definitive host of these worms.

The general structure of tape-worms should be known, and the differences indicated in the tabular statement of the well-known human tape-worms will then be understood.

The tape-worms consist of a head or fixed portion attached by hooks or suckers, or both, to the intestinal wall. This "head" is called the *scolex*. From this scolex growth takes place continuously in one direction; at first as a narrow neck which is not segmented, but which rapidly becomes segmented, and as growth continues each segment increases in size and becomes sexually mature. Each segment is known as a *proglottis*. When sexually mature the eggs are fertilised and finally the genital organs atrophy and the proglottis is reduced to a muscular sac distended by a uterus filled with fertilised eggs. These proglottides become detached and are passed in the stool. Each proglottis is motile and may live for some time after it has been passed in the stool. It creeps about discharging its eggs. These eggs are taken up by the intermediate host, another mammal, a fish or even an insect, and develop in that animal, the intermediate host, into the cystic or larval stage. In the case of some of the tape-worms, as in *Bothriocephalus*, a ciliated embryo is formed which swims freely in water, and in its intermediate host does not form a cyst but an elongated, worm-like larva known as a "*Plerocercoid*."

If taken, with food or otherwise, into the intestinal tract of man, the cyst is set free and the head becomes the scolex of the mature tape-worm. This scolex fixes itself to the intestinal wall and gives rise to the proglottides by growth from it.

The tape-worm derives its nutriment by osmosis from the intestinal tract. There is no intestine and no trace of one. There are water vascular tubes, the water vascular system running the whole length of the worm. With this exception, and the nervous system, each segment or proglottis is a distinct individual jointed on to its predecessor and successor.

The points in the structure of a proglottis are best observed in a half-grown proglottis, as earlier the organs are not fully developed and the last segments are merely muscular egg sacs with atrophic organs.

For permanent specimens the method to be adopted is as follows: Stain for twenty-four hours with very weak borax carmine; soak in glycerine for some months. Compress between two slides clamped together and place in methylated spirit. When partially hardened the pressure can be relaxed and the specimen dehydrated in alcohol. Clear with oil of cloves and mount in balsam. Pressure should be applied to the cover-glass till the balsam has hardened.

The proglottis is covered with a transparent cuticle and has a powerful muscular wall with longitudinal and transverse or circular bands. In the interior of the segment are the organs of generation, male and female, as each segment is hermaphrodite. The arrangement of these organs varies greatly in different species, but they conform to a common type.

The space between the organs is occupied by parenchymatous tissue in which are often included highly refractile calcareous masses which must not be mistaken for eggs.

The male genital organs consist of a number of small testes. Minute *vasa efferentia* unite about the centre of the body into a common vas deferens, this terminates in the copulatory organ or *cirrhus* opening with the vagina into a genital cloaca.

The female genital organs consist of the vagina leading as a straight tube from the genital cloaca into an enlargement, the receptaculum seminis. From this the tube is continued to the shell gland, and near it the ovarian tube, or tubes, if, as is usual, the ovary is paired, open. There is a diverticulum running longitudinally in the centre of the proglottis, which at first is simple but later branched—the *uterus*. The continuation of the vagina

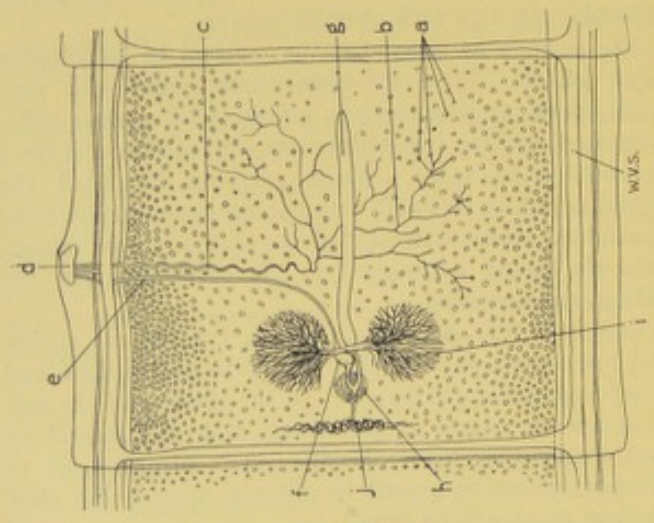

FIG. 111.

a, Testes; *b*, vasa efferentia; *c*, vas deferens; *d*, genital pore; *e*, vagina; *f*, receptaculum seminis; *g*, uterus; *h*, shell gland; *i*, ovary; *j*, vittellarium or yolk glands; *w.v.s.*, water vascular system.

is surrounded by the shell gland and the duct of the *vitellarium* or yolk gland opens into it.

The spermatozoa pass up the vagina and the eggs discharged from the ovaries are fertilised, receive their

yolk and shell, and are then forced into the longitudinal diverticulum or uterus. As more and more eggs pass into the uterus this tube becomes distended and the lateral diverticula enlarged, and ultimately the whole proglottis is occupied by the uterus distended with ova.

The projection marking the genital cloaca, into which

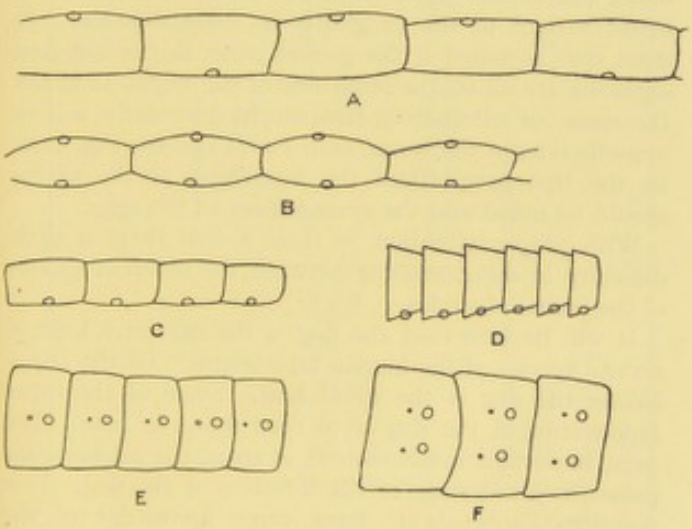

FIG. 112.

a, Tænia Saginata ; b, Dypilidium ; c, Davainea ; d, Tænia Africana ; e, Bothriocephalus ; f, Diplogonoporus grandis.

both the male and female organs open, is known as the genital pore (fig. 111).

In examining a tape-worm the points to observe are :—

(1) The size, shape and number of proglottides in the worm.

(2) The size of the scolex and its armature, which may

be suckers only, or suckers and hooks, and the number of these.

(3) In the proglottides the relative length and breadth of the segments, particularly of the mature ones. The number of genital pores in each proglottis—two in *Dipylidium* and *Diplogonoporus*, one in most of the other genera. The position of the pore, which is marginal in most, but in the mid-ventral line in the middle of the broad surface of the proglottis in *Bothriocephalus*. It must also be noted if the genital pores in the different segments are all on the same side of the worm, as in the *Davainea*, or alternately (frequently irregularly so) on opposite sides of the worm, as in *Tænia saginata* (fig. 112). In the ripe proglottides the branching of the uterus should be noted and the arrangement of the eggs.

With proper attention to these points there is little difficulty in differentiating between the different species of the human cestodes.

It will be seen that the dog is the definitive host of several species of the human tape-worms. Of the *Dipylidium* the dog is the usual host. Some of the other tape-worms of the dog have been found in man. The most important cystic cestode of man, the *echinococcus*, passes its adult stage in the intestine of the dog. It is therefore important to have some knowledge of the canine tape-worms. The subjoined table by Henry B. Ward gives the leading characteristics of the best-known of these.

Trematodes or flukes are rarely met with in man outside the Tropics. Of the human Trematodes one, *Distoma pulmonale*, is found in the lungs; another, the *Bilharzia* (*Schistosoma*) *hæmatobia* occurs in the blood-vessels. Other trematodes in the liver, and the eggs only, which are passed down the bile ducts, are found in the fæces, and still others are found in the intestinal tract, so that ordinarily the eggs, and after the adminis-

- Four suckers on the head
  - Head armed; genital pore marginal and
    - Single
      - Numerous proglottides. Strobila several centimetres long. Segments
        - Never more than slightly broader than long. Small hooks with guard
          - Bifid; hooks
            - 230-260 mm. long, genital pore very salient. *Tænia serrata.*
            - 136-157 mm. long, genital pore not very salient. *T. serialis.*
          - Entire; large hooks
            - 180-220 mm. long, length of mature segments double that of their width. *T. marginata.*
            - 150-170 mm. long, length of mature segments treble their width. *T. cœnurus.*
        - Much broader than long, except the distal segments, which suddenly elongate; genital pore usually large and prominent. *T. krabbei.*
      - Three or four segments some millimetres long ..... *T. echinococcus.*
    - Double and bilateral ..... *Dipylidium caninum.*
  - Head unarmed. Sexual orifices on the ventral surface ..... *Mesocestoides lineatus.*
- Two suckers on the head; genital pores on the ventral surface ..... *Dibothriocephalus fuscus.*

tration of powerful anthelmintics, like thymol, the adults also of these are passed by the rectum.

The Trematodes include the *Distoma*, which are flattened bodies, of oval shape with pointed ends; from the peculiarity of their shape they are popularly known as "flukes." They are hermaphrodite, non-segmented and possess an incomplete intestine. They are armed with two suckers placed near each other, anterior and posterior in most of the genera.

In the *Amphistoma* the suckers are at opposite ends of the body and they differ in shape from the other Trematodes.

The intestinal system of the Trematodes consists of a short muscular pharynx leading from the anterior sucker longitudinally. The œsophagus terminates by bifurcating into the two cæca which pass round the body towards the posterior extremity of the worm. These cæca end blindly, but are often sacculated or have diverticula. The genital organs are complicated and the arrangement varies. In *Bilharzia* (*Schistosoma*) the male and female are distinct and the female lives in an incomplete canal, the gynephoric canal in the male. In the other Trematodes the male and female organs are contained in the same animal, but the openings of each are distinct.

The female organs consist of a convoluted uterus opening externally near the second or ventral sucker.

This convoluted uterus leads to a dilatation surrounded by the "shell gland," and into this the ovarian tube from the single ovary opens. The common vitelline duct formed by the junction of the two vitelline ducts which receive the yolk from the numerous yolk glands distributed along the edges of the animals opens with it.

There are two compound testicles which lie one in front of the other.

The ducts, *vasa deferentia*, from these pass forwards

and open into a dilatation, the *vesicula seminalis*, the duct from which leads to the penis which opens externally close to the female genital opening near the ventral sucker (fig. 113).

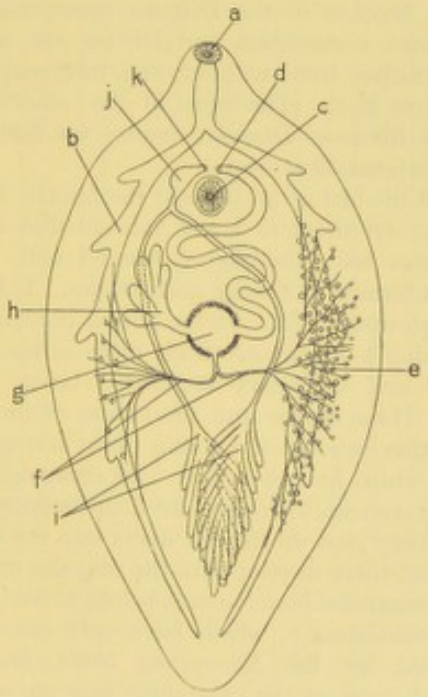

FIG. 113.

a, Anterior sucker; *b*, cæcum; *c*, posterior sucker; *d*, opening of uterus; *e*, yolk glands; *f*, vitelline ducts; *g*, shell gland; *h*, ovary; *i*, compound testicles; *j*, vesicula seminalis; *k*, penis.

The details of the arrangement vary greatly. Fertilisation is probably by a different worm. The fertilised eggs are passed with the fæces, sputum, urine, &c., of the definitive host.

The structure of *Distoma* is best shown as in the case of the cestodes by prolonged immersion in glycerine and then passing through alcohol and oil of cloves. They can be stained lightly with weak borax carmine before they are placed in the glycerine.

In one division of the *Distoma monogenia* the eggs develop into a condition suitable for the invasion of their definitive host without the intervention of an intermediate host. The eggs of the other and more important division, *Digenia*, require for their development an intermediate host.

The full life-history of none of the human Trematodes is known. Of those in lower animals that in *Fasciola hepatica* has been thoroughly worked out. A ciliated embryo is formed in the egg and escapes. It then passes into a fresh-water snail. In the snail it becomes hollowed out, forming a *sporocyst*. Buds form in the interior of this cyst and secondary flagellated larvæ, *rediæ*, are formed. These escape into the tissues of the snail, and by a further process of budding form tertiary larva or *cercariæ*, which have a sucker, and escaping from their host are taken up with grass by their definitive host, the sheep. They pass up the bile ducts into the liver of this animal and there develop into the sexually mature form.

The Trematodes found in man belong to four families :—

The *Monostomidæ*, which have only one sucker, are represented by the *Monostoma lentis*, found in the superficial layer of the crystalline lens on one occasion only.

The *Fasciolidæ* have two suckers, one terminal and the other ventral. Of this family four genera are found in man : *Dicrococlini*, in which the testicles are in front of the female genital organs ; *Opisthorchis*, in which the testicles are behind the female genital organs ; *Mesogonimus*, in which the genital opening is behind the posterior sucker ; and *Paragonimus*, in which the genital

	Length	Breadth	Suckers	Situation	Other Hosts	Geographical Distribution
Fasciola hepatica	20-30 mm.	8-13 mm. ...	The ventral sucker is much the larger	Biliary canals ...	Sheep and other animals. Man very rarely	Widely distributed.
Dicrocoelium Lanceolatum. (*Distoma lanceolatum*)	4-9 mm. ...	1·5-2·5 mm.	Ventral sucker slightly larger	Biliary canals ...	Sheep. Very rarely man	Widely distributed.
Opisthorchis Felineus ...	7-18 mm. ...	2-2·5 mm....	Ventral sucker slightly smaller than the terminal one	Biliary canals ...	Dog, cat. Found in man once	Siberia.
Opisthorchis conjunctus. (*Distoma conjunctum*)	9-12 mm. ...	2-5 mm. ...	Ventral sucker smaller ...	Biliary canals ...	Fox, dog, man	India.
Opisthorchis sinensis. (*Distoma sinense*)	10-15 mm.	2-3 mm. ...	Anterior sucker much the larger	Biliary canals ...	Man	China, Japan, India.
Opisthorchis buski. (*Distomum crassum*)	35-75 mm.	14-20 mm.	Anterior sucker the larger. The two suckers are very near each other	Small intestine...	Man	India.
Mesogonimus heterophyes. (*Distoma heterophyes*)	1-1·5 mm.	·7 mm. ...	Anterior sucker very small	—	Man	Egypt.
Paragominus Westermani. (*Distoma ringeri or pulmonale*)	8-16 mm. ...	4-8 mm. ...	Both small	Lungs	Man	China, Japan.
Amphistomum hominis ..	10-12 mm.	5-6 mm. ...	Posterior sucker much the larger. The genital pore is nearer the anterior sucker	Large intestine	Man	India.

opening may be median or right or left of the middle line.

The *Amphistomidæ* have two suckers, both terminal, one at the one end and the other at the other end of the animal (fig. 114).

Schistosomidæ have two suckers, but the male and female organs are in separate animals.

The Nematodes found in the human intestine are the *Ascaris lumbricoides*, *Oxyurus vermicularis*, *Uncinaria duodenalis* (*Anchylostomum duodenale*), *Trichocephalus dispar*, *Trichina spiralis*, *Strongyloides intestinali* (*Anguillula intestinale*).

Ascaris lumbricoides. These are large round-worms. The males are 15 to 17 centimetres in length and 2 or

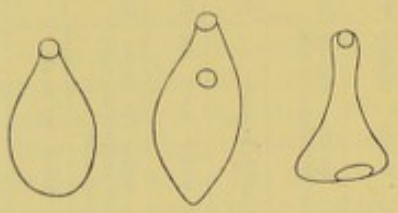

Fig. 114.

3 mm. in breadth. The female is rather larger, 20 to 25 centimetres long and 5·5 mm. in breadth. These worms are found in any part of the intestinal tract and occasionally pass through the common duct into the gall-bladder or even the biliary ducts. They have been found in hepatic abscess. They may be very numerous in the intestine.

Oxyurus vermicularis is a small cylindrical worm which tapers towards the tail. The male is 3 to 5 mm. in length and at the tail is coiled up in a spiral. The female is 9 to 12 mm. in length. This worm is found in the large intestine and rectum and may escape through the anus (fig. 115).

Trichocephalus dispar (whip-worm). The characteristic of this worm is a long, thin, anterior portion somewhat resembling the lash of a whip. The male is 35 to 45 mm. in length, and the female 35 to 50 mm. These worms are found commonly in the cæcum and also in the ascending and transverse colon. They are very rarely found in the ileum (fig. 116).

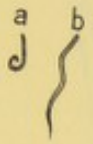

FIG. 115.
a, Male ; *b*, female.

FIG. 116.
a, Male ; *b*, female.

The *Ankylostomum duodenale* or *Uncinaria duodenalis* is of the greatest importance. These worms are found in the small intestine and may be very numerous. Both males and females are found. They fix themselves to the intestinal wall and live on the blood they imbibe. The female adult worms are 7 to 15 mm. in length and ·8 mm. in breadth. They have a mouth surrounded by

a powerful armature consisting of two pairs of curved teeth on the posterior wall of the opening and of two

FIG. 117.
a, Male, ; *b*, female, magnified ; *c*, male ; *d*, female, natural size.

triangular plates terminating in sharp points anterior to the mouth. The intestine is nearly straight and com-

consisting of two pairs of curved
wall of the opening and of two

FIG. 117.
ngnified. c, male; d, female, natural size.
minating in sharp points anterior to
estine is nearly straight and com-

mences as a powerful œsophagus
terminal. The genital opening is
of the body.

The males are rather smaller in
and ·5 mm. in breadth; they hav
The caudal extremity is expande
fold of the integument divided in
of which the lateral ones on each
There are two equal spicules wh
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hatch out either whilst still in th
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body are capable of full developme
and reproduction.

Trichina spiralis.—The adult fo

mences as a powerful œsophagus. The anus is subterminal. The genital opening is posterior to the middle of the body.

The males are rather smaller in length, 6 to 11 mm., and ·5 mm. in breadth; they have similar mouth-parts. The caudal extremity is expanded into a membranous fold of the integument divided into four unequal lobes, of which the lateral ones on each side are the largest. There are two equal spicules which can be protruded through the cloaca (fig. 117).

Another species closely resembling this is described by Guiteras in Havana. The differences are mainly in the smaller size of the head and mouth. The armature differs as there is only one pair of curved teeth.

The ankylostome is supposed to gain access to the body by the mouth, but it has been shown to be capable in its embryonic form of penetrating the skin, and some experiments seem to show the possibility of these embryonic forms obtaining access to the intestine after penetration of the skin.

The ankylostome eggs hatch quickly, within forty-eight hours, and the embryos rapidly increase in size. If kept in the fæces they soon die, but if allowed to escape into the earth they undergo further development, become sexually mature, and may reproduçe altogether outside the body.

Strongyloides intestinalis, *Anguillula intestinalis*, or *Rhabdonema intestinale*.—This is a small worm only 1 mm. long and 50 μ in breadth. It is found in the small intestine and the male is not known. Only a small number of eggs are formed, four or five as a rule. The embryos hatch out either whilst still in the adult or when discharged into the intestine. The embryos outside the body are capable of full development to sexual maturity and reproduction.

Trichina spiralis.—The adult forms are found only in

the intestines of man and other animals. They are found only for a period of a few weeks after eating flesh in which encysted embryos were present. The cysts are dissolved in the stomach, the embryos are set free, and in the human small intestine pass through several metamorphoses and become sexually mature in a few days.

The mature worms are just visible with the naked eye. The male measures 1 to 5 mm. in length and ·04 mm. in breadth. There is a straight intestine with a powerful œsophagus. The anus opens into a terminal cloaca into which also the vas deferens from the single testicular tube opens. There are two digitiform appendages, one on each side of the cloaca, which serve as copulatory organs.

The female is larger, 3 to 4 mm. in length and ·06 mm. in breadth. There is a single ovarian tube which is continued into a widely-dilated portion, the uterus, from which a narrow tube leads to the genital opening which is situated about the junction of the anterior fourth of the body with the rest.

The embryos are passed alive but do not appear in the fæces as they pass through the intestinal wall, and finally reach the muscles and there become encysted.

Man can therefore be both the intermediate and definitive host, but many other animals harbour the parasites, particularly pigs and rats. It is from badly-cooked pork that man usually becomes infected.

To find the adults the intestinal contents should be examined drop by drop with a low power. They may be found by examination with a simple lens, but are easily overlooked. The encysted embryos are seen as white specks in the muscles and are most numerous towards the insertion of the muscle. They may be found in fat and other tissues, but are less easily seen in such situations.

Protozoa belonging to various orders are found in the stools. Of these the *Amœba coli*, a large amœba which

is passed with the mucus in many chronic and recurrent dysenteries, is of the most importance. Some observers state that it is found in normal stools, and it is certainly found in stools of patients who do not complain of either diarrhœa or dysentery. In a large proportion of these cases it will be found that mucus is passed with each stool and in some ulceration of the colon has been present and found on *post-mortem* examination.

In a fresh stool the diagnosis is easy, particularly in a warm tropical country or where a hot stage is used. The large cells with active amœboid movement often containing in their interior red corpuscles vacuoles, permit

FIG. 118.

of no mistake. If the stool has been some time passed, allowed to cool or treated with antiseptics, diagnosis is less easy, as the amœba when they die become globular and are not easily distinguished from other large cells in the mucus. If they contain blood corpuscles or other substances taken as food they can be more readily recognised. They stain well with any basic stain, but there is no satisfactory differential stain.

The life-history of the amœba has not been conclusively demonstrated, and the pathogenic properties are disputed by many. In the most severe cases of dysentery the *Amœba coli* is not found, and it is usually absent in epidemic dysentery.

Amœbæ coli are found in the pus of hepatic abscesses. They are very difficult to find in the pus discharged at first. If the pus be examined three or four days after the abscess is opened they are usually readily found.

Coccidia are said to have been found in human fæces.

Various flagellated organisms have been described in the stools. The most important is a *Cercomona hominis* (fig. 118). It is a small round body with one or two long flagella. It is rarely found in healthy stools but may be common in some cases of diarrhœa.

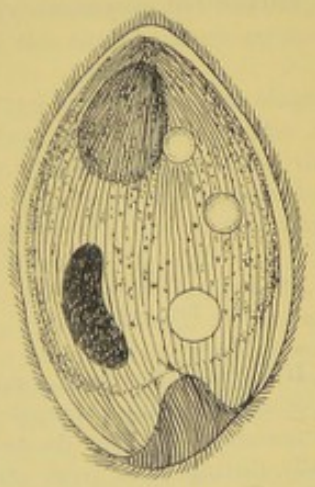

Fig. 119.

Flagellated organisms have also been found in the mouth and in abscesses in connection with mouth cavity.

Infusoria are found in some cases of diarrhœa; the best known resemble a large *Paramœcium—Balantidium coli*. It measures 65-85 μ in length. It may be found in very large numbers in the stools, and in such cases it may also be found in the intestinal walls and even in

the blood-vessels; it has been found in the pus of an abscess of the liver. It is probably pathogenic. It is a parasite found very commonly in the intestines of pigs (fig. 119).

Vegetable micro-organisms abound. Most of these belong to the *coli* group and include organisms which are harmless and others which are pathogenic. Many of the organisms, as for instance the *Bacillus coli communis*, though harmless to persons in good health as long as they are contained in the alimentary canal, can, under certain circumstances, invade the tissues and then become actively pathogenic and in some cases pyogenic. The intestinal mucosa possesses considerable power of resistance even to many decidedly pathogenic organisms, and consequently attempts at infection by the imbibition of cultures, &c., often fails.

Impaired resistance due to bad health, malnutrition, combined with enhanced virulence of an organism, is necessary in many cases for even pathogenic organisms to cause disease.

The isolation and identification of pathogenic and non-pathogenic organisms in the alimentary canal is a matter of considerable difficulty and complexity on account of the large number of organisms and species of organisms usually present.

CHAPTER XIV.

Urine.

It is not proposed to consider the ordinary tests for the abnormal constituents of urine, albumen, sugar, and the like, but only a few special points in connection with urine examination in the Tropics.

Blood is found under special circumstances as a result of parasitic invasions by *Filaria nocturna* and *Bilharzia hæmatobia* respectively, and there is at least one form of tropical hæmoglobinuria—blackwater fever.

Hæmaturia can be easily distinguished from hæmoglobinuria by the presence of red corpuscles in the deposit. In many cases it is easily distinguished with the naked eye as the superjacent fluid may not be coloured with blood in hæmaturia. If coloured with blood it is cloudy and not a clear and transparent red as the solution of hæmoglobin is in hæmoglobinuria.

In hæmaturia from bilharzia infection the bulk of the urine is often free from blood, but if the patient, after apparently emptying the bladder, strains the last few drops, blood may be found or mucus, and in this blood or mucus the characteristic ovum with its terminal spike and ciliated embryo will be found.

In all infected cases it is therefore necessary for the patient to strain, and the few drops so passed are the most important for examination.

The bilharzia has been found not only throughout Africa, in Arabia and Cyprus, but also recently in one

of the smaller West Indian Islands, and it is therefore possible that it may become a more widely diffused disease than is at present the case.

In most cases of bilharzia there will be a history of occasional attacks of hæmaturia. In these cases, by finding the ova in the last few drops of urine expressed from the bladder, the causation of the disease can be determined.

Hæmaturia from filariasis is rarely an admixture of blood only. More often chyle is also present and this frequently occurs without any admixture with blood (chyluria). Coagulation of the chyle and blood frequently takes place so that clots of blood-stained substance, or of pure blood, are formed, or the whole mass may set as a pinkish jelly.

The embryos of the *Filaria nocturna* may be found in the urine, but are more abundant in the blood. If scanty any small masses of blood or filaments of thread should be examined as the filariæ often adhere to them. Some authors advise filtering the urine, and in the last few drops left in the filter the embryos will be found. There is no periodicity in the appearance of the filaria in the urine.

If filariæ are not found in the urine they may be found in the peripheral blood of the patient if the examination be made at night.

Hæmaturia may also result from other causes, such as calculus, malignant disease, but those are not limited to the Tropics.

Hæmoglobinuria, or the passage of urine coloured with dissolved hæmoglobin, is the characteristic of "blackwater fever." Cases of paroxysmal hæmoglobinuria would, no doubt, if they occurred in an endemic area, be mistaken for blackwater fever. Hæmoglobinuria is met with in Africa as a common disease, in some places 10 per cent. of the most susceptible population (Euro-

pean) are attacked annually, or one quarter of that proportion of the less susceptible Asiatics. In some parts of India a fair number of cases are met with, but only in small proportion as compared with Africa. Cases are reported from other malarial countries, South America, West Indies, South of Europe, &c., but the disease is rare in those countries.

The urine when first passed is clear and, when diluted sufficiently, transparent, but as it cools, and particularly when it becomes alkaline, a thick deposit is thrown down.

The greater the dilution required to render the urine transparent the more concentrated is the hæmoglobin solution in the urine, and the larger the amount of the hæmoglobin the more severe will be the attack.

Rate of Secretion.—In this and also in yellow fever the rate of secretion of the urine is a matter of great importance, as if the urine is much diminished the prognosis is grave and active measures are urgently required. The times of micturition and the amount passed each time must be observed, and amount of urine passed at a micturition divided by the times in terms of an hour will give the rate of secretion per hour. Any fall in this rate is an important warning. If suppression is once established recovery will not take place in either disease.

Bile in the urine may occur in some cases of malaria as a transient phenomenon. The persistent presence of bile in an acute attack of malaria is rare, but a serious and usually fatal complication.

There are cases of jaundice occurring in the Tropics associated with high fever which are neither yellow fever or malaria. These require investigation. Nothing is known of the true nature of these diseases.

Hæmoglobinuric urine can be distinguished from bilious urine by dilution, when the red colour of the hæmoglobin is seen. By shaking the urine and noting the pink tinge of the froth as compared to the yellow

tinge of the froth of bilious urine, the distinction is readily made.

The most satisfactory method for diagnostic purposes is the use of a spectroscope, when the hæmoglobin bands will be clearly seen (*vide* Table of Spectra, Chapter V., p. 132). In some of the cases all through, and in others at onset and end of an attack, methæmoglobin is passed alone. Such urine is a brownish colour and can only be distinguished by the spectroscope (spectra 4 and 5). There is reason to believe that many mild cases of blackwater fever are overlooked as the urine contains only this methæmoglobin. In this disease casts are often present in large numbers. The casts are granular, do not often include epithelial cells, but generally contain granules of bright yellow pigment derived from the hæmoglobin. Such casts are found for weeks after an attack of blackwater fever though the urine is free from albumen.

It is important to be able, in watching a case, to form an estimate of the variations in the amount of hæmoglobin present. This is readily done if the first urine be diluted in a test tube to a convenient known extent. This is the standard and the other urines found are similarly diluted till they match the standard.

Indican is very commonly present in the urine of patients in the Tropics, usually in cases of intestinal disorder. It is best detected by conversion into indigo blue. The simplest method is to place a crystal of potassium chlorate on the bottom of a tube and cover this crystal with the urine. Strong hydrochloric acid is allowed to run down to the crystal without mixing with the urine. A blue ring forms at the point of junction of the two fluids if indican be present.

Bacteria are frequently met with. Of the pathogenic organisms the warnings which will be given as to the danger of confus.ng the smegma bacillus with tubercle

must be borne in mind. The typhoid bacillus may be found in the urine for prolonged periods after recovery from the disease.

The bacteriology of the urine in the Tropics has received little attention.

It is well to remember that urine can be used as a medium for growth of organisms. Typhoid and many others grow fairly well. It requires boiling, filtering, sterilisation, and can be used either as a fluid medium, or by the addition of gelatine or agar made into a solid medium.

CHAPTER XV.

THE pathogenic micro-organisms of vegetable origin have not been so much studied in the Tropics as elsewhere, and much of the easier work could be done without complicated apparatus or any great difficulty.

The methods now employed in laboratories require too much apparatus and are too complicated to be used by a private worker in the Tropics, and therefore he can only advance as far as is possible with the simpler methods which are at his disposal. This account of the methods which can be used is therefore intended only for those obliged to use primitive methods and makeshifts.

For the isolation and cultivation of vegetable micro-organisms artificial media are necessary, and the basis of the standard media is nutrient broth. There is much difficulty attending the making of nutrient broth from meat in the Tropics, but meat extracts, particularly Bovril or Liebig's, make an efficient substitute, and in broth prepared from either of these the organisms that will grow in nutrient broth made direct from meat will grow fairly well.

An iron enamelled jug, measures, scales and weights, a glass rod, a funnel, and ordinary filter paper or white blotting paper is all the apparatus required. Bovril, peptone, and common salt and water are the substances needed, and litmus paper, or better, phenolphthalein, which is required for the neutralisation of the broth when made, as well as a carbonate of soda or sodium hydrate solution.

Nutrient Broth.—To make the broth: Take 1,000

cc. or 1 litre of water; then take 5 grammes each of Bovril (or Liebig) and salt, and 10 grammes of peptone (Wittes' is usually used). Mix the peptone with about 25 cc. of the water and stir it well so as to form a kind of emulsion; then to this add remainder of the water and the salt and Bovril. The Bovril can be conveniently weighed in a watch-glass, or if Liebig is preferred, this can be spread with a spatula on a piece of filter paper, and the watch-glass with the Bovril in it, or the filter paper with the Liebig's Extract on it, can be placed in the water with the other ingredients. The whole should now be boiled for a quarter of an hour to ensure thorough solution and well stirred. It is now ready for neutralisation. When made with Liebig the broth will be much too acid to get good growths, and with Bovril, though much less acid, is still too acid to be quite satisfactory. Moreover, the degree of acidity of different specimens varies.

Neutralisation.—Litmus paper can be used in an emergency to determine the reaction of the broth, but is unsatisfactory, as many of the organic acids do not affect litmus paper, and the dibasic sodium phosphates act on litmus paper as an alkali. Many specimens of broth also have a double reaction, turning red litmus paper blue and blue litmus paper red, so as to leave the point of neutralisation uncertain.

Where possible phenolphthalein should be used. A ·5 per cent. solution of phenolphthalein in spirit is the index. This solution is colourless when acid or neutral, but turns a deep magenta colour with any free alkali.

Carbonic acid should be expelled by boiling from a measured quantity of the broth, say 25 cc.; to this a few drops of the phenolphthalein should be added, and then drop by drop the alkaline solution, till the broth turns a flesh or faint pink colour, indicating that the alkali is completely neutralised. The amount of alkaline

solution has been measured, and as there are 975 cc. of broth left the amount required for the neutralisation of the 25 cc. multiplied by 975, will give the amount of the alkaline solution required for the neutralisation of the broth.

It is to be noted that to exactly neutralise the broth it is of no importance what the strength of the alkaline solution may be.

A neutral broth so prepared will serve for the growth of most organisms, but the best growths are obtained with a slightly alkaline broth. If it be desired to use a less or more alkaline broth it is necessary to have an alkaline solution of known strength.

The solutions used are the so-called "normal solutions." A normal solution is a solution of the equivalent weight in grammes of the substance dissolved in water up to 1,000 cc. A decinormal solution is one-tenth of that strength or the equivalent weight in grammes dissolved in water up to 10,000 cc. A centinormal solution is the same weight dissolved in 100,000 cc.; whilst a dekanormal solution is ten times as strong as the normal or the same weight dissolved in 100 cc., *e.g.*, the equivalent weight of sodium hydrate, NaOH, is $23 + 16 + 1 = 40$, of sulphuric acid, H_2SO_4, as it neutralises two molecules of sodium hydrate, is $\frac{1}{2}$ $(2 \times 32 \times 64)$ or $\frac{98}{2} = 49$.

A normal solution is represented by $\frac{n}{1}$, a decinormal by $\frac{n}{10}$, a centinormal by $\frac{n}{100}$, and a normal solution of sodium hydrate therefore is 40 grammes dissolved in water and diluted to 1,000 cc., whilst a normal solution of sulphuric acid will be 49 grammes diluted to 1,000 cc.

A neutral broth is one which is neutral when tested hot with phenolphthalein; such a broth is usually alkaline when tested by that uncertain standard litmus paper. The degree of alkalinity of a broth is measured by the number of cc. of normal alkaline solution added per

1,000 cc. of broth over and above that required for neutralisation, the minus sign — is used to indicate the alkalinity, so that — 4 would indicate that of a solution $\frac{n}{1}$ of alkali 4 cc. added to 1,000 litres of the broth in excess of the amount required for neutralisation.

If the broth used is still acid as tested by phenolphthalein, that is indicated by the plus sign +. A broth described as + 10 would still require the addition of 10 cc. of $\frac{n}{1}$ solution of alkali per litre for neutralisation. Many specimens of Bovril broth, without neutralisations, are not more acid than this, and + 10 is a favourite reaction for the growths of many organisms.

This question of neutralisation and of uniformity of reaction is a simple matter. The degree of alkalinity or otherwise of the media affects the properties of growths so materially that it is necessary to be particular on the point, but for mere growth of organisms a broth neutral to phenolphthalein will suffice.

After neutralisation or procuring the required degree of alkalinity or acidity to phenolphthalein, the broth should be boiled and kept at the temperature of boiling water for half an hour. It should then be allowed to cool, as it is not till it is cold that the mass of the phosphates will be precipitated. It is then, whilst cold, to be filtered through ordinary white filter paper. The broth is now prepared, but in the course of the preparation many organisms will have gained access to it from air, vessels, &c., and if left as it is these would multiply. Sterilisation is therefore necessary. This can either be done in bulk or the broth can be decanted into a series of test tubes in quantities suitable for use.

The procedure differs little in the two cases. If it be desired to keep the broth in bulk it should be poured into a clean narrow-necked vessel (Erlenmeyer flask, fig. 120), which will stand heat, and the mouth of this

vessel plugged tightly with cotton-wool. If it is to be divided, some 10 cc. should be poured into each of a series of clean test tubes and the mouth of each should be plugged with cotton-wool. It is better to sterilise by dry heat the flask or the tubes and wool before pouring in the broth. It is not absolutely essential, as the tubes, wool, and broth contained in the tubes can all be sterilised together, but if not failures are likely to

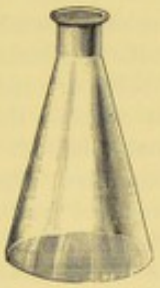

FIG. 120.

occur and many of the tubes will befound to be contaminated with organisms.

For sterilisation a single boiling does not suffice, as some of the organisms and most spores are only slowly killed at the temperature of boiling water.

Sterilisation.—To sterilise, the broth and the vessels containing it should be maintained at the temperature of boiling water for at least half an hour on three consecutive days and allowed to cool in between. This intermittent method allows the spores which have escaped the first sterilisation to develop into the less resistant organisms before the second heating, which then destroys them. The third sterilisation, which is not always absolutely necessary, is a precaution in case any spores or organisms have escaped from the two previous sterilisations.

Storing Media.—The broth when cool is ready for use and can be kept till required. The tubes, wool, and broth are all sterile and remain so for a considerable period. Organisms can only gain access to the broth by growing through the wool. This does not take place through dry wool, but in moist, warm climates, such as are met with in the Tropics, the wool gets damp and growth through it takes place.

In such climates and at such times of the year it is advisable as a routine every week or fortnight to heat the end of the test tube containing the cotton wool so as to ensure the wool being dry and to kill any organisms that have grown in it. Unless this precaution is taken tubes soon become contaminated.

The number of organisms falling on to the cotton-wool can be greatly reduced by covering the top of the tube with an inverted paper cone, such as a folded filter paper. This measure would delay the contamination of wool even in the Tropics.

Glycerine Broth, &c.—For many purposes additions are made to the nutrient broth. These additions must be made before sterilisation; if made after, the sterilisations will require to be repeated. They are best made before neutralisation.

Glycerine broth is made by the addition of 6 per cent. of glycerine. Glucose, lactose, maltose or saccharose, added in the proportion of 2 per cent. to the broth, make glucose broth, lactose broth, &c., respectively.

Solid Media.—For other purposes, and those of the highest importance, the broth is mixed with gelatine or agar-agar in sufficient proportion for the solution to set when cooled to the temperatures at which it is desired to study the growths. These form the so-called *solid media.*

Nutrient Gelatine.—The gelatine medium is made by the addition of 9 to 12 per cent. of the best French

gelatine to the crude broth. Broth that has been neutralised and filtered can be used, but it is waste of time as neutralisation will have to be repeated. Gelatine is always acid.

After the gelatine has been added in the required proportion keep in the steamer for half an hour; neutralise, render alkaline or leave acid to the required extent. Allow to cool to 60° C. or less as long as the gelatine remains fluid.

Whip up the white of an egg for each 500 cc. of the gelatine broth and mix well with the rest of the medium. Steam for half an hour. The white of the egg diffused through the medium will coagulate, and in its coagulation will carry down many of the impurities of the gelatine. Filter whilst hot through a coarse filter paper—Chardin's—which should be moistened with hot water. Store in flasks or decant into test tubes as required. Sterilise as with nutrient broth for half an hour on three consecutive days. This medium is known as nutrient gelatine, or simply "gelatine."

Glucose, &c., &c., can be added to it if required, the addition being made preferably before neutralisation and always before sterilisation. Too prolonged heating causes hydrolytic changes in the gelatine so that it will not set. Extra sterilisations must be avoided where possible on this account.

Nutrient Agar.—Agar, nutrient agar, is made in a similar manner; 1·5 to 2 per cent. of the powdered agar is added instead of the gelatine. It is much more difficult to filter, and where the necessary trouble cannot be taken a passable substitute is to allow it to cool slowly so as to permit the coagulated egg albumen and other precipitates to settle to the bottom. When cold the mass can be removed from the vessel and the lower part containing the great mass of the impurities cut off. The residue, though much inferior in appearance to the

filtered product, is sufficiently clear to be translucent and can be satisfactorily used without filtration for cultures.

The clearer filtered product is better. To filter it is necessary that the filter paper should be kept hot. This is best effected by placing the funnel, filter paper, and receptacle in the steam steriliser and allowing the filtration to take place in the steam steriliser.

Addition of glucose, &c., can be made as in the case of other medium.

Filtration of these media is facilitated by folding the filter paper so as to have a large number of angles and very little of the paper in contact with the glass. These papers can be bought ready folded or can be folded before use.

The solid media are essential for the separation of the various organisms usually present in the animal tissues, discharges, or other substances to be examined.

Separation of Organisms.—The method of procedure is based on the principle that by successive dilutions of a minute quantity of the substance to be examined the individual organisms will be so scantily distributed through the medium that they will be separated from each other by an interval appreciable to the eye. If this medium be then allowed to become solid the organisms will remain well separated from each other, and if kept under conditions favourable for their growth will multiply and form in the course of a few days "colonies" which will be visible to the naked eye. From these colonies sub-cultures can be made, and the colonies may also be examined directly.

A piece of platinum wire, 3 to 4 inches in length, is inserted into the fused end of a glass rod, and a small loop is made at the other, the free end, of the platinum wire. This wire is sterilised by heating in the flame, and a loop-full of the substance to be examined is taken up by this loop.

A tube containing the gelatine medium melted by placing in hot water is then inoculated with this loopful and well stirred and shaken.

The amount of the substance is thus diluted by the amount of the fluid gelatine.

After sterilising the needle a loopful from this tube is inoculated into a second tube and will again be diluted to the same extent. A third tube is treated in the same manner and the dilution will now be extreme.

In other words, provided the mixing is thorough the organisms will be so much diluted by these successive dilutions that they will be separated from each other by appreciable intervals. A fourth or a fifth dilution may be made, but is not usually required, as the third dilution is in most instances sufficient.

FIG. 121.

The end of each of these tubes is heated in turn to destroy any organisms which may be present at the edge of the tube with the plug withdrawn, and the gelatine is poured into a flat sterilised glass dish (fig. 121) —a Petri dish—which is quickly covered with another similar but larger sterilised dish. The melted gelatine solidifies as a thin sheet of nutrient gelatine, and is allowed to remain at a temperature of about 20° to 22° C.

Some organisms will grow quickly and others slowly, and by colour, size, shape of colonies and effect on the gelatine it is usually possible to distinguish that several organisms are present. In the plate from the first tube

the colonies are so numerous that they are separated from each other by too small a distance to isolate. In the plate from the second the organisms may be sufficiently far apart, and in the third and subsequent dilutions the colonies resulting from the growth of the widely-separated organisms are usually far enough apart to be easily distinguished from each other. From these cultures can be made.

As the first dilution is always too little diluted for practical work and the second is usually so, it is unnecessary to do these dilutions in the solid medium or to make plates of them. The two first dilutions may be done in sterile broth or even in a weak sterile salt solution, 5 grammes to a litre, and only the third in the solid medium. This economises the solid medium, which is the most troublesome to prepare.

Plating may be done with agar, but a thermometer must be used to make sure that the agar is cool enough, otherwise the organisms may be killed. The agar will have to be heated to nearly the boiling point of water to become thoroughly fluid, and allowed to cool before inoculation. It is not so easy a proceeding as plating with gelatine, but agar is the only solid medium that can be used in many parts of the Tropics, as above 22° C. the gelatine will not set. Stronger solutions, as 20 per cent. gelatine, will remain solid at 37·5° C., but these stronger gelatines are not easy to work with and frequently undergo changes during sterilisation that cause liquefaction or acid production. Therefore, unless ice and a cold incubator are available we are restricted to the use of agar for plating.

A convenient method of plating in agar is to make the agar plates and inoculate when the agar has set either from the second or third broth dilution, by making a series of parallel strokes with a platinum loop on the solidified surface, or, and better, by using a sterilised

brush—camel's hair—and brushing lightly over the surface of the medium after dipping this brush in the second or third broth dilution. Excess of fluid is to be avoided by draining off from the brush against the inner side of the tube. The brush should be sterilised in a dry tube, plugged with wool, by three successive sterilisations. The platinum wire is sterilised as usual by heating in the flame.

Some important organisms will not grow on any known artificial medium, and others only on special media or under special conditions. Separation of such organisms is either impossible or difficult. Standard books on bacteriology should be consulted for such methods, which will not usually be practicable for the solitary practitioner under the conditions of tropical life and work.

Description of Organisms.—Having obtained a pure culture of an organism the more important points to determine are as follows :—

(1) Size, shape and arrangement. Morphological appearance.

(2) Motility.

(3) Spore formation.

(4) Anatomy. Flagellæ, capsule, &c.

(5) Staining reactions : (*a*) Simple stains ; (*b*) Gram's method ; (*c*) Ziel Nielson.

(6) Growths on artificial media : (*a*) In broth ; (*b*) on gelatine ; (*c*) on agar.

(7) Conditions : (*a*) Essential to growth ; (*b*) favourable to growth ; (*c*) inimical to growth.

(8) Chemical products : Gas formation and curdling of milk ; acid or alkali formation ; indol formation.

(9) Reaction with blood sera, particularly with the blood sera of patients suffering from definite diseases.

(10) Pathogenic properties.

In organisms such as that of lepra, which cannot be

cultivated, only a few of these points can be determined; and the pathogenicity has not been proved experimentally, with a doubtful exception, as lower animals are insusceptible. It is *inferred* from the constant association of the organisms, identified by their staining reactions and appearances in the lesions resulting from the disease, and from the observation, that they are not found except in this disease.

We propose to take briefly the methods of observing these various points. The descriptive terms used with reference to them, the relative value and the limitations to the value of each point for diagnostic purposes will also be considered.

(1) The size and shape of an organism is best observed in stained specimens, and any simple stain combined with a mordant will suffice. Films should be made on a slide or cover-glass. For this purpose with a culture in broth all that is required is to spread a drop with the platinum wire. If the culture is on agar or gelatine it should be rubbed up with a little water (sterile), and of this emulsion a portion spread out with a needle.

Preparation of Films.—If it be desired to make a film or smear from any natural fluid or excretion it may require the addition of a little water to make a satisfactory smear. From blood thick films may be used and decolourised by removing the hæmoglobin with sterile water. Tissues should be cut with a sterile knife and the cut surface rubbed on the slide.

The most difficult films to make are those such as sputum containing much mucus. The most satisfactory method is to use two slides and warm both. A portion of the mucus is transferred to one slide and this is warmed over a Bunsen flame or spirit lamp, and a second slide is warmed at the same time. The second warmed slide is used to rub the mucus on the first and is placed with the long axis at right angles to the

other, and the surfaces parallel and in contact. The slides are then separated and both are again warmed in the flame with the smeared side of each uppermost. They are again, whilst still hot, rubbed together with the smeared surface of the two in contact. This process is repeated till the films are nearly dry, when they are finally rubbed together harder. Good thin, dry films are easily and quickly obtained by this method.

However the films are made they require fixation. This is best done by heat and is usually accomplished by passing through a smokeless flame three times, the smeared side always uppermost. Do not char the film.

As many films are so thin that they are difficult to see when dry, it is well to mark the smeared side of the slide with a grease pencil. The staining fluid is simply placed over the film for the requisite time and then washed off. Stains used are Löffler's blue, five to ten minutes; carbol thionin, five minutes; carbol fuchsin or gentian violet for organisms that do not take other stains deeply, one to ten minutes.

Schizomycetes.

Definition.—The bacteria or *Schizomycetes* are unicellular vegetable organisms. Spores may be formed but not in specially differentiated cells. This separates the bacteria from moulds.

Hyphomycetes which form spores in specially differentiated cells. There is no sexual method of reproduction. Reproduction is by fission, not by budding as in the yeasts or *Blastomycetes*.

Morphology.—The various shapes of bacteria usually described are *Cocci* or rounded or oval bodies, with the greatest diameter not more than twice the least. Micrococci is an alternative term. If division takes place in one direction only the organisms may remain attached in pairs, *Diplococci*, or in chains, *Streptococci*, if a series remain attached.

In other cases division takes place in two directions, and we then find the organisms arranged in squares of four or multiples of four. Such growths are called *Tetrads*. Others divide in three directions at right angles to each other and form cubical masses, these are known as *Sarcinæ*.

A common arrangement is irregular growth in all directions, leading to an irregular mass or cluster of cocci, the *Staphylococci*.

Bacilli are cells that are rod-shaped. They are longer, at least twice as long, as they are broad, and the shorter forms are distinguished from oval cocci by having the two sides parallel. By fission they may grow into long jointed rods—streptobacilli.

Curved organisms are, when short, known as *vibrios*, when longer and more twisted as *Spirilla* or *Spirochæta*.

Leptothrix.—Rod-shaped, filamentous forms showing differentiation between base and apex, but not branching.

Streptothrix.—Filamentous forms showing true branching. These form the connecting link between the *Schizomycetes* and the *Hyphomycetes*.

Measurements are made as for other minute bodies. Some organisms readily change their form with variations in the condition under which growth has taken place. If the variations are great the organism is described as "pleomorphic." Slight variations occur in all organisms, so that morphological characters alone are not to be relied on.

(2) *Motility*.—This can only be observed in living cultures, though it can be inferred for organisms which are shown to have flagella. The motion of motile organisms must be clearly distinguished from the oscillatory movement—Brownian movement—common to all minute particles suspended in fluid.

It is best observed in a "hanging drop" preparation.

This is made by making a thick ring with vaseline on a slide and taking a clean cover-glass, rather longer than this ring, and placing near the centre a small drop of the culture of living organisms to be examined. The slide is then taken up and turned so that the vaseline ring is directed downwards, and is gently brought into contact with the cover-glass so that the drop of culture on the cover is in the centre of the ring of vaseline. The cover will adhere to the vaseline ring and form a sealed chamber, and when the slide is turned over again the drop will hang from the lid of this chamber, the cover-glass, and can then be examined. If the temperature is so high that the vaseline runs, lard can be substituted for it.

The organisms are colourless and transparent and difficult to focus, so that the light must be reduced by nearly closing the iris diaphragm. Either "$\frac{1}{6}$ or $\frac{1}{12}$" oil immersion objective may be used. It is well to focus first on to the edge of the vaseline ring and then move the slide towards the drop, keeping the droplets of water of condensation which usually form on the under surface of the cover-glass in focus till the edge of the drop is reached. With a little practice and a dim light the organisms can then be brought into focus and the presence or absence of automatic motility determined.

This property, though an important point of difference between some organisms that closely resemble each other morphologically, is subject to considerable variation, and the degree of motility in motile organisms varies from slight causes, such as slight difference in temperature, reaction of the medium, &c.

(3) *Spore Formation.*—All the micro-organisms reproduce by fission, but some of them also enter into a resting stage—spores. This resting form is much more resistent to agencies, chemical, heat, &c., which destroy organisms, so that spores will withstand for some time

temperature of boiling water, though the active phase of the organism is at once destroyed.

Some organisms form spores very readily, others only under certain circumstances not thoroughly understood, and many pathogenic and other organisms do not form spores under any known circumstances.

The spores can often be recognised in the living culture, as they are usually more highly refractile as well as rounder. For the demonstration in dried films advantage is taken of the fact that spores stain with greater difficulty, but retain their stain, when stained, better than the organisms from which they were derived. A simple method is to stain with warm carbol fuchsin for five minutes. This much overstains both spores and bacilli. Treat rapidly with 2 per cent. sulphuric acid; this, if done *rapidly*, will leave the spores stained, but remove the stain entirely from the bacilli. Wash well to remove the last traces of acid and counterstain with Löffler's blue for ten minutes, or carbol thionin. The spores will be stained by the fuchsin and the bacilli blue by the methylene blue or thionin.

This method is successful for most spore-forming organisms.

(4) Certain points in the anatomy of organisms are sufficiently definite to be of use in diagnosis.

Some organisms have a thick capsule which does not stain deeply with basic stains, or not at all.

The simplest method of demonstration is to stain the film with carbol fuchsin and to examine *in water*, not in Canada balsam. The organism is surrounded by a clear space, and the capsule with a defined edge can usually be seen.

Welch treats the film with 2 per cent. acetic acid, which causes the capsule to swell and enables it to take the stain, and then after removal of the acid stains with aniline gentian violet for five to thirty seconds. Cap-

sulated organisms often lose their capsules in culture, but the presence or absence of a capsule, as seen for instance in sputum, is of value.

A cell wall is probably present in all the organisms, but it is difficult to demonstrate. In some, however, it is fairly well marked. It is best shown after the cell contents have been caused to shrink by salt solutions or iodine solution (plasmolysis).

Flagella.—Most motile organisms have been shown to have flagella. They are variable in number, and whilst the vibrios have usually only one or two the motile bacilli may have large numbers. The number of flagella is of some value in the differentiation of species, and the presence, absence, or plan of arrangement is of differential value in grouping organisms.

The methods of demonstration cannot be considered as satisfactory or easy, and there is considerable uncertainty in the results; they are all troublesome. The two common methods successfully employed are *Muir's modified Pitfield* and *MacCrorie's*.

By Muir's method the mordant employed is composed of :—

Tannic acid 10 per cent. aqueous solution	10 cc.
Corrosive sublimate saturated aqueous solution	5 cc.
Alum saturated aqueous solution ..	5 cc.
Carbol fuchsin	5 cc.

This is well mixed, allowed to settle, and the clear fluid decanted off and centrifugalised. This mordant keeps for about a fortnight, but must be centrifugalised every time before use.

The stain employed is composed of a saturated solution of alum, 25 cc., with 5 cc. of alcoholic gentian violet saturated solution. This must be prepared immediately before use.

The smears should be made from agar cultures, twelve to eighteen hours old, emulsified with a little distilled water. Spread very gently on a cover glass, freshly flamed to free from grease. Hold in Cornet's forceps (fig. 122), being careful that the film side corresponds to the fenestrated side of the forceps, otherwise mistakes as to which is the film side may occur.

Allow the film to dry in air and fix by passing through the flame. Cover with the mordant and heat till it steams for two minutes. Wash well in water and dry carefully. Pour on the gentian violet stain and heat till the staining fluid steams for two minutes. Wash in water, dry and mount in Canada balsam.

Fig. 122.

This method requires the use of a centrifuge, but gives a large proportion of successful results.

MacCrorie's method is simpler. A single stain combined with a mordant is used for staining the flagella and the bacilli are counterstained with carbol fuchsin.

The stain is composed of :—

Night blue saturated alcoholic solution	10 cc.
Potash alum saturated aqueous solution	10 cc.
Tannin 10 per cent. aqueous solution..	10 cc.

Gallic acid 1 or 2 grammes improves the solution.

Films from young agar cultures are prepared as above and the stain is placed on the film for five minutes slightly warmed. It must be flushed off with running water or a thick dirty deposit will be left. Counterstain with carbol fuchsin, dry and mount.

(5) *Differentiation by Methods of Staining.*—The three main methods of diagnostic value are first the effect of simple stains. There is great variation in the ease with which different organisms take up stains, and this difference is sometimes of value. Some organisms do not stain uniformly, and such differences as preferential affinity of stains for the ends of a bacillus, bi-polar staining, is one of the characteristics of the plague bacillus. In cultures organisms frequently lose their characteristic staining reactions.

Of greater value are two special methods.

Gram's method is based on the fact that some organisms will retain their stain when treated with alcohol if, after staining, they are treated with a solution of iodine. Such organisms as retain the stain when treated by Gram's method are said to "stain by Gram." A freshly-prepared solution of gentian violet in aniline water is made by shaking up a few drops of aniline oil with water and filtering. To this is added drop by drop an alcoholic solution of gentian violet till a metallic film begins to form on the surface. With this stain the fixed film is stained for five minutes. (If now treated with alcohol the stain would be completely removed from all the organisms.)

In some organisms the addition to the film of Gram's iodine solution, composed of iodine, potassium iodide, and water, for two minutes, will fix the stain in these organisms so that when the film has been treated with alcohol they still retain the purple colour, whilst it is removed from everything else. Such organisms are said to "stain by Gram."

The alcohol is kept on till it ceases to remove any more colour and not longer, as in time it will remove the stain even from the organisms which stain by Gram.

It is convenient instead of using a plain alcohol to use an alcoholic solution of eosine ½ per cent., as then

organisms which do not stain by Gram will be stained faintly by the eosine. This is of most use when working with a mixture of organisms, such as is met with in many secretions, &c.

For sections it has the additional advantage that it does not require such prolonged treatment with alcohol as is required if alcohol is first used to decolourise and then again for counterstaining.

Most of the pyogenic cocci, the organisms usually found in suppuration, stain by Gram. Many of the organisms associated with intestinal and other diseases do not stain by Gram.

A comparatively small number of groups of organisms are described as *acid fast*, because when once stained they retain the stain even after treatment with fairly strong, 25 per cent., solutions of the mineral acids. Hydrochloric or sulphuric acids are those used.

The method employed is to use a strong basic stain such as fuchsin in a 1 in 20 solution of carbolic acid in water, and either to stain in the cold for some hours, or more conveniently to warm until the carbol fuchsin steams, and then keep warm for five minutes.

This is conveniently done on the slide. The film is fixed as usual and covered with the carbol fuchsin. The slide is placed on a copper which has been warmed in the flame and left there, fresh stain is added if evaporation is too rapid or the stain shows signs of boiling.

The stain is flushed off and replaced by a 25 per cent. solution of sulphuric acid. The pink colour disappears and is replaced by a yellow. The film is again washed and if still pink again treated with sulphuric acid. This is repeated till on washing at the most a faint pink colour returns.

The specimen is well washed in water to completely remove the acid and counterstained with Löffler's blue for five minutes. Wash, dry, and either examine

directly by placing a drop of oil on the film or mount in Canada balsam. The acid-fast organisms retain the red colour of the fuchsin whilst other organisms are stained blue by the methylene blue which is used as the counterstain.

In tropical work it is important only to use fresh carbol fuchsin. The solution keeps well in England, but in the Tropics it deteriorates so that sometimes in a week or so, and at others in some months, it ceases to stain well.

The more important members of the acid-fast group cannot be cultivated in the simpler media. It will therefore be convenient to consider these organisms here. There are four main groups of the acid-fast organisms, which will be considered under the heading of the best-known member of the group :—

(1) Tubercle ; (2) lepra ; (3) smegma ; (4) Timothy grass.

Some forms of the *Streptothrix* group are also "acid-fast."

(1) The tubercle group includes the organisms found in tuberculosis, in mammals, birds and reptiles.

The organisms can be cultivated on blood serum and nutrient glycerine agar, or in glycerine veal broth. Growth is slow and much affected by the temperature. The preferential temperature is that of the animal from which the organisms were obtained.

The mammalian, avian and reptilian tubercle bacilli therefore grow at different temperatures and are only pathogenic to mammals, birds and reptiles respectively. Some authorities hold that they are modifications of one and the same organisms and that they can, by suitable methods, have their characters altered so that the differences disappear. By most authorities the three are considered to be

specifically distinct, and some go further and do not admit the specific unity of the tubercle organisms in different mammals. Koch holds that bovine and human tuberculosis are distinct on the ground that their pathogenicity varies.

Tubercle bacilli in man are found in the secretions or excretions from an infected organ and therefore may be found in sputum, urine, &c. They usually set up a granulomatous new growth which has a marked tendency to caseate and break down. The organisms may be in large numbers, but in some situations, such as the skin, bones, &c., they are usually found only in small numbers. They are found but rarely in the blood.

(2) *The Lepra Bacillus* is the only representative known of this group. It cannot be cultivated on artificial media, and all experiments at inoculation of lower animals have failed. The organisms are found in extraordinary numbers in leprous tubercles in the skin, and when these ulcerate, in the discharges from these ulcers. Before ulceration the bacilli can be readily demonstrated, by compression with a clamp of a tubercle or portion of infiltrated skin. On pricking this, serum loaded with the bacilli will exude. The bacilli are not found in ulcers or sores in purely nerve leprosy, as in that form they are present in the nerve sheaths and the ulcer or necrosis is not due to the breaking down of a leprous granuloma or of tissues infiltrated with the organisms.

One of the most constant natural discharges to contain the bacilli is the mucus discharged from the nose. It is also one of the earliest manifestations in many cases, including some of nerve or anæsthetic leprosy. By some it is believed that the earliest and most constant lesion of leprosy of

all forms is a deposit in or below the nasal mucous membrane.

Various differences in size, staining reaction, &c., between lepra and tubercle bacilli have been described, but they are not sufficiently marked or constant to be of diagnostic value. The most important diagnostic point in films of mucus in leprosy is the aggregation of the bacilli into small, dense clumps, in many cases still retaining the outline of the cell in which they grew. In sections of skin the extraordinary profusion of the organisms, as well as the aggregation into compact masses, is characteristic of lepra bacillus as opposed to human tubercle. In some of the lesions of avian tubercle a similar grouping may be found.

(3) *Smegma Bacilli.* — This group probably includes several species. In most specimens of smegma the organisms, though truly acid fast, are decolourised by alcohol, that is, they are not alcohol fast. Some varieties, however, do not lose their stain in alcohol and are, like the tubercle, both acid and alcohol fast. This organism has been the cause of frequent mistakes in diagnosis, as urine easily becomes contaminated with this bacillus, and it may readily be mistaken for that of tubercle and a diagnosis of urinary or renal tuberculosis given. In the majority of cases the use of alcohol as well as of acid will prevent this mistake, but as some specimens of the smegma, including Lustgarten's so-called syphilis bacillus, are also alcohol fast, the possibility of the confusion should be avoided by using the catheter.

(4) The fourth group, of which the Timothy grass bacillus, or *B. Phlei*, is taken as the type, are the only organisms of this group which grow readily on almost any medium. They are found on several

species of grass used as fodder and may be found in enormous numbers in the fæces of cattle. As a consequence they are often found in milk and products, such as butter and cheese, derived from milk. Several varieties or species have been described. This group is of economic importance, as cattle have been condemned as tuberculous on the grounds that acid-fast bacilli were found in the stools and milk; butter and cheese have also been condemned on this insufficient reason. These organisms are not pathogenic.

(6) With organisms that can be cultivated the growths on artificial media, nutrient broth, gelatine or agar, differ in some cases sufficiently to be of diagnostic value, and in any case the character of the growth is one of the properties of an organism that requires description.

Cultures may be made on plates as in the separation of different organisms, or, and more conveniently, in tubes. The growths in fluid media are made by taking on a sterilised platinum loop a portion of the culture and inoculating the tube. The nature and character of the naked-eye appearance in the broth at varying periods should be described. The temperature at which the cultivation is made must also be noted where incubators are available. Blood heat 37° C. and 21° C., are the most convenient, and terms like "room temperature" should be avoided. In many tropical countries the temperature of the air will range from 25° to 30° C., and satisfactory growths of the more important organisms can be obtained. In a description of the growths at these tempertaures a result intermediate between those at incubator temperatures will be obtained. All cultivations when made must be carried on in the dark.

The points to be observed in a broth culture are the surface, whether covered with or free from a film or

pellicle. In the body of the fluid note if the fluid is turbid and the degree of turbidity, if not turbid whether quite clear or with floating particles; the presence or absence of a precipitate and, if one be present, whether it is composed of a uniform fine deposit or if in separate masses. Any change in colour, and bubbles from formation of gas, must be further noted.

On solid media the growths may be observed on plates or in tubes. In tubes the growths can be seen on sloped cultures by drawing the inoculated platinum loop over the surface of the medium obtained by placing the tube, whilst the medium was still liquid, in a sloped position and allowing it to set, or in stab cultures. In these the medium is allowed to set with the tubes vertical. An inoculated wire, not a loop, is plunged steadily into the depths of the medium and withdrawn without splitting the medium.

The appearance of the separate colonies is most important. There are great diversities in the appearance of growths on solid media, and an accurate series of defined terms for descriptive purposes is much needed. Such a series of descriptive terms has been drawn up by Chester, but many of the terms will probably not be generally accepted and they are used at present by few bacteriologists.

In any description the points to be noted and described are the size of the individual colonies, their shape, the character of the edge, their elevation, whether raised or depressed, and a detailed account of the character of the surface. The microscopic appearances of the colony: if transparent, whether highly refractile or not, and if not clear whether opalescent, finely or coarsely granular, or irregularly blotchy. Any colouration, either of the colony itself or the surrounding medium, must be noted. In some organisms the different colonies remain distinct even when in contact, whilst with other organisms adjoin-

ing colonies readily grow together or become confluent. In growths on gelatine the presence or absence of signs of liquefaction in the surrounding medium is a point of the first importance.

In stab cultures any surface growth must be noted, as well as the more important growth in the line of puncture.

It may be uniform, finely or coarsely granular, composed of numerous fine or coarse colonies which remain discrete and are not confluent, or of large masses.

Extension into the gelatine in the neighbourhood of the puncture may take place, and the character of these extensions, whether as knots or as fine filaments, or in an irregular arborescent manner, is worthy of attention.

If liquefaction, in a gelatine medium, has taken place it will be well shown. It is usually most abundant in the upper part of the line of puncture, but with organisms that grow best in the absence of air will be more conspicuous in the depths.

Air bubbles along the line of puncture, indicating formation of gas and any colouration of the growth or of the medium, must be noted.

The amount of growth that takes place in a given time as compared with other organisms, or similarly the relative amount of liquefaction, gas formation, &c., in the time, is an aid in distinguishing allied or similar organisms, though liable to be modified with different strains of the same organism.

There is no cultural characteristic that cannot be modified by frequent subculture, culture under different conditions of temperature, reaction of medium, and other influences. The cultures of some organisms vary more than those of others. The information gained as to the character of growths, therefore, though of considerable value, has to be considered with other properties of the organisms.

Cultures on milk, potatoes, &c., are of more diagnostic value in cases of some special organisms.

(7) One of the conditions affecting growth of micro-organisms, the effect of oxygen, is of special practical value.

Some organisms will only grow in presence of oxygen; such organisms are strictly *aërobic*. Others will not grow at all in the presence of oxygen, these are said to be strictly *anaërobic*. The largest number of bacteria are intermediate between the two and are termed facultative anaërobes.

Aërobic organisms grow readily under the ordinary conditions, as even in stab cultures there is usually sufficient oxygen present for the commencement of growth.

Anaërobic organisms are most easily grown in stab cultures of glucose, agar or gelatine, or in glucose formate agar made by adding ·02 per cent. sod. formate to glucose agar (Kitisato). The tubes must have been freshly boiled to expel air from the medium, and the stab should be made with a fine needle so as to carry down as little air as possible. After the needle is withdrawn the upper part of the medium should be heated so as to melt it and seal the opening made by the needle.

It must be remembered that though growth of anaërobic organisms does not take place in presence of air, the organisms, and particularly the spores, may retain their vitality and grow if transplanted into more favourable conditions.

(8) The chemical products of organisms vary both with the nature of the organism and the character of the medium. Gas formation is one of the easiest to determine, but it is also necessary to have in the medium some substance from which the gas is to be formed. The sugars are valuable for this purpose, and it will be found that whilst one organism will form gas from either

glucose or lactose another will form gas only from glucose.

Another manifestation of chemical activity is the formation of acid or alkali.

Formation of acids and gases are of particular importance, as so many of the organisms found in the intestine either form gas and acid from glucose or form acid only.

The formation of gas can be shown in most stab preparations as bubbles of gas form along the needle track. It is better shown by melting the gelatine, or, better, glucose gelatine, and rotating the tube alternately in opposite directions after inoculation. Such a preparation is known as a "shake culture," and after it has set and grown bubbles of gas will be formed all through the medium.

A better method that can be used with fluid media is to place in the medium a small inverted tube, Durham's tube. During sterilisation of the medium the gas will be expelled from this small tube which will be completely filled with the fluid medium. If the tube be inoculated with an organism that forms gas from the medium the small inverted tube will catch the gas as it ascends and, according to the amount contained in the tube, will float above the surface.

Gas formation from glucose is one of the characteristics of some of the commoner intestinal bacteria.

Acid formation can be shown by using a neutral or slightly alkaline medium coloured with litmus; the formation of acid is shown by the change in colour of the litmus.

Most of the intestinal bacteria form acid readily. An ingenious application of these properties of the intestinal organisms is that of MacKonkey for the detection of fæcal contamination of water, milk and other substances. He uses Durham's tubes and employs a medium con-

taining bile salts. Bile salts inhibit the growth of many organisms, but are favourable to the growth of intestinal bacteria.

The medium he employs is composed of: Peptone, 20; salt, ·5; sodium taurocholate, ·5; water, 100; to which is added glucose or lactose in the proportion of ·5 per cent. The medium is neutral and is coloured with neutral litmus. A Durham's tube is placed in the test tube containing the medium and during the three sterilisations required will be filled with the medium.

A measured amount of the water, &c., to be tested is added and the tube incubated at preferably 42° C. for twenty-four hours. All the organisms so far tested, which in this medium produce acid and gas, are inhabitants of the intestinal tract.

Those which form acid only are mainly, but not entirely, pathogenic or non-pathogenic intestinal organisms. Of the other organisms many will not grow in the medium at all, or in the medium at the temperature of incubation, or if they do grow form neither acid nor gas.

If, therefore, neither acid nor gas is formed the evidence is strong that there is no living fæcal contamination. If acid alone is formed it is doubtful whether there is such contamination. If acid and gas are both formed there is strong probability that the water, &c., is contaminated with organisms that are inhabitants of the intestinal tract.

Indol formation is another important chemical product of some bacteria. A simple medium is required, and plain peptone water made by boiling 10 grammes of peptone and 5 grammes of salt in a litre of water is the medium usually employed. This should be filtered and sterilised as usual.

The tube of this medium should be inoculated with the organism to be examined and incubated for at least twenty-four hours. Other tubes inoculated at the same time are incubated for longer periods.

To this culture a little pure sulphuric acid is added; a red colour developes in a few minutes if *indol* and a *nitrite* have been formed.

If the colour remains unaltered, three or four drops of a ·05 solution of sodium nitrite should be added to the mixture, and if a red colour now developes indol alone has been formed. Yellow-forming nitric acid which contains traces of nitrites may be satisfactorily used instead of nitrite.

Amongst other chemical products are ammonia, alcohol, phenol, sulphuretted hydrogen, and the substances which cause curdling of milk.

(9) Certain pathogenic organisms effect a change in the blood serum of persons infected with these organisms, so that the serum contains substances which, when mixed with a living culture of the organism, cause loss of motility of the bacteria, and also cause them to aggregate in little clumps or masses. This aggregation is called agglutination, and the serum which causes this *agglutination* is said to contain *agglutinins*.

The application of the test is simple. The blood serum, free from red corpuscles, is obtained as has been already described and diluted with sterile broth to a known extent, ten, twenty, thirty or more times. Sometimes the blood is sent in capillary tubes or the serum has not well separated. In such cases to obtain clear serum it is necessary to use the centrifuge (fig. 123). This diluted serum is mixed with an equal volume of a living active culture of the organism to be tested and the mixture examined as a hanging-drop preparation. Loss of motion of the organisms and agglutination should take place within a certain time limit and a control made by using the same dilution of normal blood serum with more of the same culture. This action of the serum on the organisms is specific and affords a means of proving the co-relation of the organism and disease.

It is true that strong undiluted normal serum will cause in some cases a similar agglutination, but not with the great dilutions which will act in serum from a person with disease.

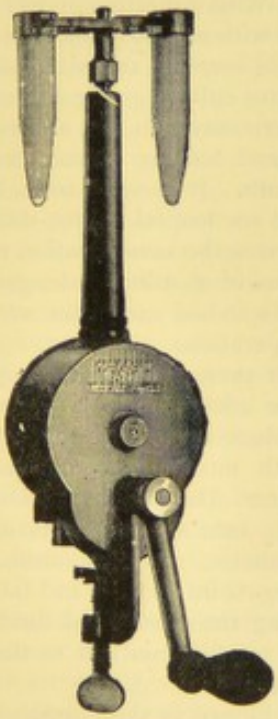

FIG. 123.—CENTRIFUGE.

The converse of this test is to use a culture of an organism to test the serum reaction of a patient suspected to be suffering from the disease which the organism can cause. Typhoid and Malta fever are the disease in which the action is most decisive. This application

of the principle is known as the agglutination test, or the Grünbaum-Widal or Widal reaction.

In the application of the hanging-drop method the serum may be diluted in Wright's tubes or by a number of loopfuls of broth being added to a loopful of serum and well mixed. With a dilution of 1 in 20, one loopful of serum is mixed with nine loopfuls of broth, and of this mixture one or two loopfuls are mixed with one or two loopfuls of an active culture giving a dilution of 1 in 20.

If higher dilutions, say 1 in 100, are required, it would be inconvenient and tedious to mix one loopful with ninety-nine of broth. It is easier to make a dilution of 1 in 10 and dilute one loopful of this dilution with nine of broth, which gives the same dilution more quickly.

The gradual loss of motility and aggregation of the organisms can be watched under the microscope in the hanging-drop preparation.

Many observers prefer the macroscopic demonstrations of the same effect. This is done by aspirating into a tube a mixture of serum diluted to the required extent with broth mixed with an equal quantity of active broth culture. The mixture of culture and diluted serum is drawn up into a narrow tube and placed vertically in the incubator. The organisms will lose their motility and aggregate into a mass and fall to the bottom of the fluid, leaving the superjacent fluid clear and free from turbidity, in marked contrast to the control which will still remain turbid.

This method is known as the "sedimentation test."

There are many fallacies which may occur in connection with these tests. The culture must be an active one and young. A control with serum of normal blood must be made and the dilution must be sufficient.

Some strains of the organisms agglutinate more readily than others, and even with diluted serum of normal blood agglutination may take place if the organisms are grown on unsuitable media.

The change in the serum is a persistent one, so that a positive reaction in the case of a person who has had a previous attack of typhoid or Malta fever gives no information as to his present condition.

(10) The pathogenic properties of an organism are shown by the effect of inoculating a susceptible animal with the organism in pure culture if possible. Where that is not possible, with fluid containing as few other organisms as possible.

In some instances, as in *tuberculosis*, the similarity of the lesions produced by a similar organism naturally occurring gave a ready guide to susceptible animals. In others a series of animals had to be used to find a susceptible host. Rats, guinea-pigs and rabbits are the animals most commonly used, but in other cases monkeys, dogs, cattle and horses have had to be employed. No such experiments can be made under the Vivisection Acts without a license, and in any case there are so many difficulties and fallacies that without a thorough study of these and of the methods employed the results obtained would be valueless.

Material used for injection may be :—

(1) Pure cultures of an organism.

(2) Products formed by bacteria in solution such as toxins.

(3) Fluid excretions, secretions and portions of diseased tissues.

The injections are usually made with strict antiseptic precautions into the subcutaneous cellular tissues. With fluid cultures there is no special difficulty. Cultures on solid media require to be emulsified with sterile saline solution. Solid tissues, portions of spleen, &c., should be rubbed up in a sterile mortar with a little sterile broth and then injected. Occasionally a small mass of solid tissue is inserted into an aseptic pocket made under the skin for the purpose, and the wound closed by a sealed dressing such as gauze and collodion.

In other cases the injection is intramuscular or intraperitoneal.

The results of inoculations are not always conclusive. The resulting disease, even in a susceptible animal, may show very little resemblance to the disease caused by the same organism in man or other animals.

An organism that is pathogenic may by successive cultures lose its virulence, and be with some animals non-pathogenic, whilst on the other hand, if passed through a series of animals, the virulence may be increased.

Koch's postulates are that :—

(1) The organisms must be found in the tissues, fluids or organs of the animal affected with the disease.

(2) The organism must be isolated and cultivated outside the body on suitable media for successive generations.

(3) The isolated and cutivated organism on inoculation into a suitable animal should reproduce the disease.

(4) The same organism must be recoverable from the inoculated animal.

These in the main are still considered sound, though not praticable for all organisms, as some cannot be cultivated ; for others no susceptible animal is yet known, and in some of the lower animals, though a disease is produced by the injections it bears little or no resemblance to the human disease under investigation.

One disease due to a *Streptothrix* is of special importance in the Tropics—madura foot. It occurs in India, Straits Settlements, East Africa, British Guiana, and Cuba, but is probably to be met with all through the Tropics.

Clinically it is a chronic disease which causes much swelling of a firm fibrous nature and destruction of the tissues, with the formation of sinuses discharging watery or oily-looking fluid. In some cases in this discharge, white, black or pink granules, visible to the naked eye, are found, and these are masses of the mycelium of the

fungus. In other cases these granules are very rare, and only the branching filaments of the mycelium are found; in still other cases these may also be absent, though in sections of the tissue the mycelial clumps are to be seen.

The organism is characterised by the formation of dense clumps of mycelium formed of the branching filaments of the *Streptothrix mycetoma*. The ends of the filaments at the edges of these masses degenerate and become swollen, forming the so-called clubs.

The organism will grow on any of the ordinary media, forming limpet-shaped masses.

In general character the mycetoma resembles the Streptothrix of actinomycosis, but it does not stain by Gram, does not liquefy gelatine, and the "clubs" are rounder.

The clumps take any of the ordinary basic stains, including hæmotoxylin, and either this stain or carbol-thionin is to be recommended to show the organism in sections.

The clumps of the mycelium set up changes in the tissue so that the growths are surrounded by a mass of newly-formed tissue of the granulomatous type.

At the periphery of the masses of this growth is much badly-formed fibrous tissue, and the centre is often broken down. It is in the breaking down of this granulomatous tissue that the mycelium clumps are liberated and are discharged with the fluids from the sinuses.

Fungi (*hyphomycetes*). — Of the various fungi which attack the skin and hair some are widely distributed, though more common and luxuriant in their growth in the Tropics. *Pityriasis versicolor* comes under this head. Others, like *Favus*, may be common in some places, but as in temperate climates, are of limited distribution. The commoner fungi attacking the hair of the head are unknown in many tropical countries. The only fungus

of this class at present recognised as peculiar to the Tropics is a cutaneous ringworm, *Tinea imbricata*, characterised clinically by the large size of the epidermal scales, separated, and the tendency to form geometrical patterns.

For the demonstration of the fungi causing these various affections the older method consisted in soaking the hair or scales in a 7 per cent. solution of caustic potash, which rendered the keratin clear and transparent, whilst the fungus was less affected and could be clearly seen. This method causes swelling of the fungus and spores, and therefore is not suited for the differentiation of the varieties or species of fungi.

A modification of Gram's method of staining gives more useful results but is slow. The hair or scale is stained in gentian violet aniline water for five minutes and dried with blotting paper. It is then treated with Gram's iodine solution for two minutes and again dried with blotting paper. It is then covered with aniline oil to which a little iodine has been added and left till the fungus can be seen. It should be examined from time to time under the microscope, as though the process is slow, ultimately even the fungus will be decolourised. Do not wait till all the tissue is clear, but when nearly so treat with aniline oil and clear in xylol. Mount in xylol balsam.

The points to observe are the arrangement of the growth, whether inside or outside the hair, scale, &c., the presence and the size of the spores. The nomenclature of these fungi is based on these points. According to the seat of growth of the fungus it is an ecto- or endothrix, and microsporon or megalosporon according to the size of the spores.

These ringworms are true *fungi*. The fungi are multicellular organisms composed of filaments, either simple or branched, or jointed or unjointed. These filaments are called *hyphæ*, and if they project into the air are

aerial *hyphæ*, or down into the substance of the medium they are known as submerged *hyphæ*.

They frequently form a compact mass—a mycelium—and if this form is a hard, dense mass it is known as a sclerotium. Sexual reproduction as well as reproduction by fission has been proved to occur in most members of the group.

These fungi include the ordinary moulds, and some, such as ergot, form compounds which, when eaten, are poisonous.

In addition to the cutaneous fungi which cause the true ringworms (*Tinea*), fungi may be found in mouth, ear or nose, as well as in pulmonary cavities. These are secondary growths. Occasionally, particularly in bird-rearers, who take uncooked grain in their mouths, a true pneumono-mycosis occurs.

It is possible that some cases of madura foot, the black variety, are due to a fungus and not to a streptothrix.

The tropical fungi, aerial and otherwise, have so far received little attention and should offer a fruitful field for research. Most of the fungi grow readily on nutrient media, but best if a sugar be added. Maltose is the most suitable for the fungi of the ringworms.

Many of the aerial fungi will not grow at high temperatures, such as blood heat, though they flourish at lower temperatures.

Yeasts, or blastomycetes, are frequently found in the mucous cavities and occasionally in ulcers or other skin lesions. They are distinguished from bacteria not only by the method of reproduction but also by their greater size. In some species endospores are formed, but these are multiple in each cell and not single as in bacteria.

The yeasts are of considerable interest, as alcoholic and other forms of fermentation are due to their agency.

CHAPTER XVI.

Measurements.

Measurements of the various eggs, parasites, and normal and abnormal cells are of considerable importance and are easily made.

The simplest and most satisfactory method of microscopic measurement is by drawing to scale, which can be readily done by the use of a camera lucida or drawing camera. A micromillimetre scale is used as an object, and with the microscope vertical the scale as it appears through the camera lucida is drawn on a piece of paper. Gowers' hæmocytometer slide, which is divided into $\frac{1}{10}$ mm., may be used instead of the micromillimetre scale, or any other will suffice. This drawing of $\frac{1}{10}$ mm. must be further subdivided by compasses. This gives the scale, and it must be determined for each objective. Provided that the distance of the paper from the camera lucida is constant, which is best ensured by working with the microscope vertical and the paper on the table, a scale once drawn can always be used.

The tube must always be used the same length.

To measure an object, all that is needed is an outline drawing through the same camera lucida, and the application of the scale to this drawing will give the measurements.

Another simple method is by the use of a micrometer eye-piece. On a glass disc a scale is drawn and this is placed in the eye-piece so as to be accurately in focus

by the anterior lens. The disc rests on the diaphragm, which can be moved so that the scale is sharply focussed. A measured scale is then placed on the stage. As before, Gower's hæmocytometer scale may be used instead of the micromillimetre scale, and the number of the divisions in the micrometer eye-piece which correspond to $\frac{1}{10}$ millimetre or a multiple of it with the different objectives is noted. With the tube at constant length the value of the divisions in the micrometer eye-piece so determined is constant for each objective. In measuring, the object to be examined is placed under the microscope and the measurements in terms of the micrometer scale determined, and from these the real measurements calculated.

For simple diameters the micrometer eye-piece is perhaps the most convenient, but for irregularly-shaped bodies, and particularly for such objects as filariæ, the use of the camera lucida is easier, quicker and more accurate.

By either of these methods all that is required to measure an object, once the scales are made, or the equivalent in micromillimetres of the eye-piece scale determined, is to change the eye-piece either for the camera lucida or for the eye-piece containing the micrometer scale.

Measurements may be represented as decimals or fractions of a millimetre, but in many ways it is more convenient to take as the standard $\frac{1}{1000}$ of a millimetre —a micromillimetre—usually indicated by the Greek μ.

If no scale is available to standardise the micrometer eye-piece or drawings, results by relative measurements can be taken and subsequently standardised. A convenient rough standard is the average diameter of a red corpuscle, which is about 7—8 μ.

Estimation of Corpuscles.—For the determination of the number of elements in a given volume of fluid, as

for instance the number of red or white corpuscles in blood, it is usually necessary to dilute such a fluid to a known extent so that the number of elements in any given volume can be counted.

Such a dilution may be made in a graduated pipette by drawing up a given volume of fluid and as many more volumes of a diluting fluid as is necessary, and mixing well. Such a mixture can also be conveniently made in Wright's tubes, as the absolute volume is immaterial; all that is required is any volume and definite multiplications of that volume in order to get the degree of dilution.

Common instruments used for the purpose are the pipettes of the Thoma-Zeiss hæmocytometer. These are so graduated as to give a dilution of 1 in 10 or 1 in 100, but can be used to give dilutions at intervals of 10 from 1 in 10 to 1 in 100, and in intervals of 100 from 1 in 100 to 1 in 1,000.

These tubes are convenient and the glass bead in the mixing chamber facilitates mixing and prevents the aggregation of corpuscles into masses. The diluting fluid, when working with blood, must be carefully selected according to the object to be attained. If red corpuscles are to be counted the fluid must be isotonic or hypertonic, so as to prevent the red corpuscles being broken up. Such fluids as sod. sulph. 10 per cent. are suitable.

In many cases it is convenient to count the white corpuscles at the same time, and in that case stains are mixed with the diluting fluid which stain the leucocytes and enable them to be readily distinguished. Toisson's fluid, viz., glycerine 30 cm., sodium sulphate 8 grammes, sodium chloride 1 gramme, methyl violet ·025 gramme, and water 160 cm., is very convenient for this purpose, but must be filtered each time before use. In other cases where it is not desired to count the red corpuscles and where these may render the enumeration of other elements more difficult it is better to destroy them. To

ensure their destruction it is advisable to use a more powerfully destructive agent than distilled water, and weak acetic acid is the one generally employed.

Leucocytes, &c., can be readily counted in blood only slightly diluted when treated in this manner.

However the dilution is made the next essential is to obtain a definite measured volume of the diluted fluid. This is done by having a cell, which when covered with a cover-glass has a definite known depth. It is also further necessary to be able to estimate the area of the

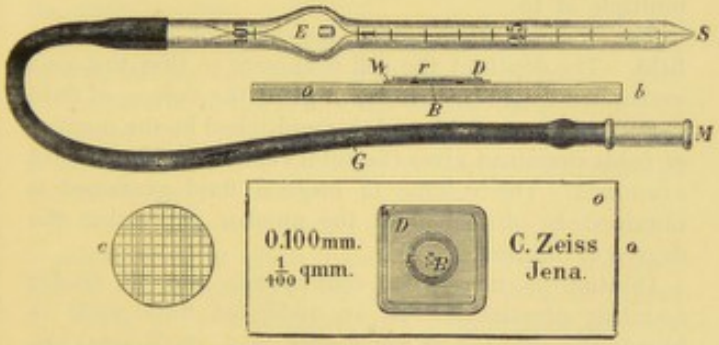

FIG. 124.—THOMA'S HÆMOCYTOMETER, BY ZEISS.

base of this cell or of the portion of it examined. In Gowers' and in Thoma-Zeiss' hæmocytometer (fig. 124) this area is determined by having the slide ruled in squares with sides $\frac{1}{5}$ and $\frac{1}{20}$ of a millimetre respectively, so that the area is obtained by multiplying the sides of the squares by each other, and this multiplied by the known depth of the cell, *i.e.*, the space between the cover-glass and slide, gives the volume of the fluid examined.

Instead of these squares others use a micrometer eye-piece ruled in squares. The size of these squares is determined by comparison with a scale under the microscope once for all.

In examining fluid for elements that are scanty it is often a saving of time to take the whole field as the area to be examined.

This area is most conveniently determined by obtaining the diameter of the field by observing the number of divisions of a scale—a hæmocytometer scale again will do—that form the diameter. To avoid fractions of a division the tube should be drawn out till the diameter is exactly a certain number of divisions and, as pointed out by Grünbaum, much calculation can be avoided by drawing the tube out till the number of divisions is a multiple of 10.

The formula then gives the area of the circular field. The depth of the cell is known, so that this area multiplied by the known depth gives the volume of fluid examined in one field, and this multiplied by the number of fields examined gives the total volume of diluted fluid examined. The volume of original fluid examined is obtained by dividing by the number expressing the degree of dilution.

Dilution is merely for convenience and to render counting practicable. In an undiluted fluid, such as blood, the number of corpuscles in a small area, say $\frac{1}{10}$ mm. square, would be some hundreds, and therefore difficult to count, but by diluting 100 times the number will be reduced in that area to a dozen or less, a convenient number for counting.

In these calculations it is well to avoid the exclusive use of formulæ. A formula is easily forgotten, or only in part remembered, and confusion and error result. If the calculations are made on general principles there is a little waste of time, but the possibility of error is avoided.

The dilution is made to a known extent—ten times, a hundred times, or so on, as is judged to be convenient.

A known volume of the diluted fluid is examined.

represented by either the area of the field multiplied by the depth of the cell, or the area of the marked squares on the slide multiplied by the depth of the cell, or the area of the square as seen in the micrometer eye-piece (previously determined) multiplied by the depth of the cell.

The number of elements which it is wished to have counted is determined in a certain number of these volumes of fluids, and the average is taken by dividing the total number by the number of volumes examined. The larger the number of volumes taken the smaller is the probable error.

All the factors necessary for the calculation are thus determined and all that is necessary is to reduce them to comparable terms so that the results obtained can be compared with other results. The number of elements is usually recorded as so many per cubic millimetres of undiluted fluid.

If the number of elements counted be X, the volume of diluted fluid in which these elements were counted is known as the volume of the square (or field) expressed in terms of a millimetre or V, multiplied by the number of these squares (or fields) N. The degree of dilution is represented by D. Then in NV of diluted fluid there are X elements; therefore in 1 cmm. of diluted fluid there are X multiplied by $\frac{1}{NV}$ elements; and as the undiluted fluid only contains $\frac{1}{D}$ of undiluted fluid, therefore the number of elments in 1 cmm. of undiluted fluid will be D multiplied by X multiplied by $\frac{1}{NV}$ or $\frac{DX}{NV}$.

This assumes that the elements are uniformly diffused through the fluid, and every effort must be made to ensure this being the case. For blood there must be no delay and the blood must be mixed with the diluent as quickly and thoroughly as possible. Many of the elements in the blood and other animal fluids are adhesive

and tend to adhere in clumps. If these clumps are present any result obtained will be unreliable.

Counts of red blood corpuscles are necessary in cases of anæmia. In some forms of anæmia the number of corpuscles is not reduced and may even be above normal. In these cases the hæmoglobin is much reduced, so that on the average each corpuscle is poor in hæmoglobin. *Chlorosis* is the type of this form of anæmia.

In others and in the Tropics, the more common form of anæmia, the number of red corpuscles is greatly reduced, sometimes to $\frac{1}{10}$ of the normal 5,000,000, and commonly to between 1,000,000 and 2,000,000. In these forms of anæmia the hæmoglobin is reduced only to about the same extent as the number of the corpuscles, so that, on the average, each red corpuscle contains about the normal amount of hæmoglobin. *Pernicious anæmia* is the type of this form of anæmia.

In the anæmia resulting from hæmoglobinuric fever the number of the red corpuscles is very rapidly reduced, so that in the course of three days the red corpuscles will fall from 5,000,000 to 1,000,000 or even less, per cmm. of blood. In malaria it is unusual to find any decided diminution of red corpuscles as the result of an acute attack of malaria, though repeated attacks may cause a considerable degree of anæmia of this type.

In *anchylostomiasis* in most cases there is a great reduction in the number of red corpuscles, so great in chronic progressive cases, that only 500,000 red corpuscles may be found in a cubic millimetre. There is often, however, an even greater fall in the hæmoglobin; so that though in the main this form of anæmia is of the type of pernicious anæmia, in some cases it is of a mixed nature.

The forms of anæmia of the pernicious anæmia type are usually due to blood destruction or *hæmolysis*. In

such forms of anæmia pigment and iron deposits are found in the cells of the liver, kidneys, &c.

Various chemical agents can cause hæmolysis and it occurs as a result of the action of various organisms and animal parasites.

The Leucocyte Counts should be supplemented by a differential count of the leucocytes in a well-stained film. By the combination of these methods the number of leucocytes of each variety in a cubic millimetre of blood can be determined.

Increase in the total number of the leucocytes occurs in many diseases; it is known as *leucocytosis*. Diminution in the number of leucocytes—*leucopenia*—is of less importance, but occurs in some stages of malaria. Leucocytosis is marked in most cases of pneumonia, and the amount of leucocytosis increases with the severity of the case to a certain extent. In the most severe attacks, however, there may be no leucocytosis, and therefore the absence of this condition is of even more unfavourable prognosis than a most marked manifestation of it.

In septic conditions, appendicitis with suppuration, hepatic abscess, septic endocarditis, &c., there is well-marked leucocytosis, though not to the same extent as in some cases of pneumonia.

A great increase also occurs in scurvy. Even in health there is a considerable variation—7,000 to 10,000—in the number of leucocytes, and daily a variation occurs owing to the increase in the lymphocytes during active digestion.

It is not usual for the variation in the number of the different forms of leucocytes to be increased uniformly. Usually some forms are increased and others either increased to a much smaller extent or even diminished.

In pneumonia the increase is mainly that of the polymorphonuclear leucocytes, whilst the eosinophile leucocytes are not only in smaller proportion but in smaller

numbers than in normal blood. After an attack of malaria the leucocytes are usually in normal number, as the larger mononuclear leucocytes are increased and the polymorphonuclear leucocytes diminished.

If in addition to the normal white corpuscles other cellular elements are present in the blood, such as myelocytes, this indicates a definite blood disease.

If two diseases, such as pneumonia and malaria, coexist in the patient the influence of the one disease appears to overpower the other, so that the leucocytic variation of pneumonia only will be present.

A variation in the total amount of blood in the body no doubt occurs in certain diseases, but there is no simple practical method of determining such variations.

To enumerate the larger parasites, such as filarial embryos, no dilution is required. A measured quantity of blood is taken up in a pipette and blown out on to a slide. This blood is spread out and allowed to dry and decolourised by placing in water. The total number of filarial embryos can then be counted and reduced to the proportion per cmm.

This is a matter of no difficulty and the only method to be relied on. Substitutes that are often employed are to make a thick dry blood film a certain size, or to use cover-glasses of a definite size in making fluid films, or to make a certain number of drops of blood in making the film. These methods as substitutes for direct measurement are inferior, and results arrived at by these methods have little value.

The use of measured amounts of blood, decolourised and stained, for the estimation of malarial parasites is not to be recommended. The results are unreliable with these parasites as so many are broken up during this process. An approximation to the number present can be obtained by first estimating the number of leucocytes present per cmm. and then taking a fluid fresh

film of the blood and determining the relative number of parasites to leucocytes in this film. If, for instance, ten parasites are found in this film and 100 leucocytes, there will be one parasite to ten leucocytes, and if the number of leucocytes determined separately is found to be 8,000 per cmm., then the number of parasites should be one-tenth of this or 800 per cmm.

The results are approximate only, as leucocytes are not uniformly distributed in the fluid film. The relative numerical proportion of the parasites to leucocytes, if determined in a dry film, is far more inaccurate, as the distribution of the leucocytes is so unequal in such a film, and many of the leucocytes adhere to the needle, slide, or paper used in making the film. The leucocytes in a thin part of the field, such as is used for the observation of parasites, will be from one-half to about one-tenth of the proper amount, and the error in counting the parasites will therefore vary from this cause to the same extent.

An approximation can also be obtained by determining the average number of parasites in a field. If the average number of red corpuscles in the same field is also determined the method is of value, but it is tedious.

In fluids other than blood, where parasites, including bacteria, are numerous and minute, Wright suggests mixing this fluid with an equal quantity of blood diluted so that the number of corpuscles per cmm. is known, The relative proportions of the parasites to the blood corpuscles, as determined by making a dried film of the mixture and staining it, will then enable us to estimate the number of organisms present in any given quantity of the fluid.

The more usual method of estimating the number of bacilli in a fluid is to take a measured volume of the fluid and add to it a measured quantity of liquefied gelatine and plate it.

The number of colonies found in this plate will give the number of organisms in the volume of fluid taken. If the colonies are too numerous the number found in any measured area may be counted, and this result reduced to terms of the total area of the plate. If the number of organisms is very great a second or third dilution, always with measured amounts of the fluid and gelatine, may be necessary.

For air determination a measured amount of air is driven through liquefied gelatine, which is plated, and the number of colonies estimated as before.

For earth or solids these must be finely divided and weighed amounts taken.

It is essential that the plates should be accurately levelled and as much of the plate as possible should be counted. Agar plates may be used, and for complete investigation incubated at various temperatures. Other plates should also be made and incubated aërobically and anaërobically.

The numerical estimation of eggs in fæces hardly admits of practical application, as the amount of water &c., in the fæces varies so greatly and eggs are not uniformly distributed. Here loose terms, such as numerous, very numerous, moderate number, or few, are more in accordance with the amount of information possible, than a numerical estimate which has a superficial appearance of exactness could be.

Colorimetric Estimations are not very accurate. They are, however, the only simple methods that can be used with rapidity and ease. They are all based on the comparison of the diluted fluid with a substance standardised as regards colour. The principle adopted is to so arrange matters that the equality in tint, or "matching," of the two objects compared is obtained, as equality in tint can be determined more exactly than degrees of difference.

In such colorimetric examinations the same source

of light must invariably be used, and for general purposes artificial, particularly candle-light, is to be preferred, as colours seen by one light will not match when viewed with another light.

The methods for blood work most used are Gowers' hæmoglobinometer and its modifications, in which gelatine, coloured with picro-carmine, is used as the standard colour and standardised so as to represent a certain

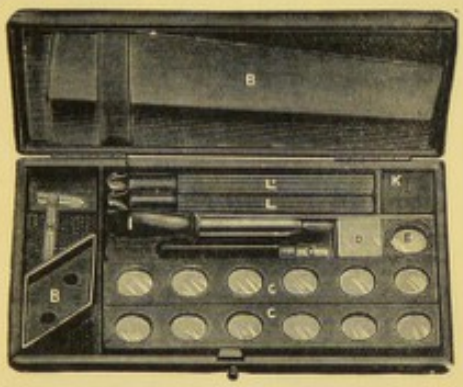

Fig. 125.—Oliver's Tintometer.

percentage — 1 per cent. — of hæmoglobin, and von Fleischl's hæmometer and its modifications, in which a wedge of tinted glass is used as the standard, this being moved till the thickness of the wedge is such that the depth of colour equalises that of a definite depth of the fluid to be examined.

Thirdly, in Oliver's method (fig. 125), a series of graded depths of coloured discs are used as the standard. The colour produced is compared with these standard colours by mixing a definite fixed quantity of blood in a glass cell of fixed depth and capacity with water placed on a white plaster disc.

For all such methods it is necessary that the blood corpuscles should be broken up and the hæmoglobin be in solution. This can be done by dilution with distilled water, or, better, by dilution with a 1 per cent. solution of carbonate of soda in water.

In Gowers' method (fig. 126) the standard colour is equivalent to that of normal blood diluted so as to represent 1 per cent. of hæmoglobin dissolved in water. The fluid to be estimated is placed in a cylinder, or, better, a flattened tube exactly similar to that containing the standard, and is diluted drop by drop till

FIG. 126.—GOWERS' HÆMOGLOBINOMETER.

the two colours are matched. The tube is graduated so that the degree of dilution can be read, and the amount of dilution necessary to produce the same depth of colour indicates the relative amount of hæmoglobin as compared to the standard.

In von Fleischl's method (fig. 127) the wedge of coloured glass is arranged on a stand and illuminated from below by a plaster of Paris disc.

Fitting into the circular opening of the stage is a metal cylinder with a glass bottom and this cylinder, is divided longitudinally into two compartments. One is filled with diluted and laked blood and the other with water.

Under this second is the wedge of coloured glass, and this wedge is moved horizontally by a rack and pinion till the colour corresponds to or matches that of the diluted blood. The movement of the wedge is indicated on a scale graduated by comparison with hæmoglobin solutions of varying strength, so that the hæmoglobin equivalent of the portion of the glass wedge in the field can be read at once.

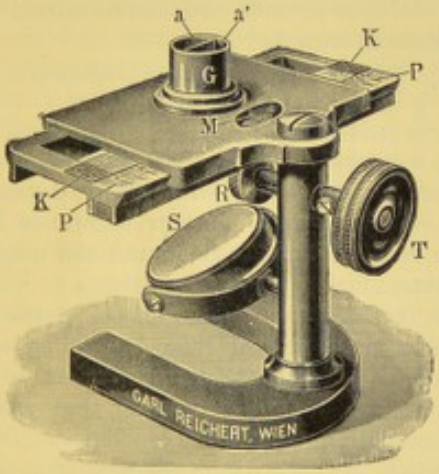

FIG. 127.—VON FLEISCHL'S HÆMOMETER.

Statistics.—Statistics as reliable as those obtainable in England can rarely be obtained in the Tropics, and an important source of information is thus removed. Even statistics of births, deaths, and the more important diseases, both of Europeans and natives, have to be admitted with great caution, and local knowledge of the manner in which they are compiled is essential before giving them even the slightest consideration. With local knowledge the statistics may be very valuable, but

even then the errors due to selection of statistics, a personal equation, greatly reduces their value.

Statistics for tropical work have therefore to be to a great extent the result of individual work, and therefore the large numbers that European statisticians are accustomed to work with are not obtainable.

It is therefore of the greatest importance at the outset that the probable error due to working with too small figures should be clearly grasped.

The probable or possible error can be calculated according to Poisson's formula :—

$\frac{m}{g} \pm 2\sqrt{\frac{2\,mn}{g\,3}}$ = possible variation in proportion of m to g.

g = total number of events.
m = number in one group.
n = number in other group, so that $m + n = g$.

This formula only deals with the mathematical relationship of the figures. Further allowance has to be made for errors of observations and the numerous uncertain factors met with in any statistics.

A mere consideration of the effects of the extent of the possible mathematical error in dealing with small figures suffices to indicate the care that is necessary and the multitude of observations that are requisite before formulating a definite conclusion. In spite of the magnitude of this error, of the numerous possibilities of errors of observation, and even of the fact that mere increase in the number of observations may only multiply the same error or doubtful point, as the same source of error may be included in each observation, the acquisition and use of statistics is of high value and often indicates a correct conclusion. The liability to error diminishes but does not destroy this value.

It is customary to indicate all results in percentages, and no doubt this method renders comparison easy, but a consideration of the formula of probable error shows that 1 in 4 is by no means the same thing as 25 in 100.

The number of observations made must be included in any account, and whenever possible these observations should greatly exceed the 100. The consideration of the magnitude of the probable mathematical error under the most favourable circumstances should lead to as great an exactitude as possible and avoidance of other and avoidable sources of error.

Value of Evidence.—Considerable judgment as well as caution is requisite in obtaining information other than that derived from personal observation.

As regards occurrence of diseases, parasites, &c., much of the information received must be taken with great caution, as it is often from laymen and untrained observers. Even more in the Tropics that in England such persons hold theories either of their own or derived from others, and are anxious to bring forward only facts which are in support of these theories.

It is well in making enquiries to be careful to limit the enquiry to points that are within the power of any ordinary observer. It is not well to discard altogether such evidence, as on many important points information can be derived, and in some of these the liability to error is no greater than with a professional observer.

Various points in connection with malaria might be well taken as illustrations, both of the value of such information and the errors that are likely to occur as a result of too much confidence in such information, as well as of the general methods which have been adopted determining etiological and other factors.

These points comprise: (1) *As regards Individuals.*—Their susceptibility to the disease and the effects of the disease, including liability to relapses, length of period of intermission between relapses and any evidence of the acquirement of immunity.

(2) *As regards the Population in General.*—Susceptibility, and any factors, age, race, or habits, influencing it.

Mortality per 1,000 of the population at various age periods; and case mortality in treated and untreated cases; liability to any special, immediate or remote, complications; effect on general health; any evidence of acquirement of immunity.

(3) *As regards the Place.*—This should include enquiries as to any special house, village or district, as well as the country in general, where the disease is more or less prevalent than the average. Seasonal variations and their effects, particularly rainfall, temperature, and any cause affecting level of sub-soil water. Any facts known as to the prevalence of the known main factor, in the case of malaria prevalence of Anopheles, in the spread of the disease. Some numerical estimate, *endemic index*, of the liability to infection.

Most of these points can be determined to some extent by careful enquiries, though the results must be confirmed by observation, or where possible by the adoption, as a check, of other methods.

The results obtained in this way, though not to be implicitly relied upon, will be a valuable guide to the direction of researches required in a district or country.

Liability to Infection.—Enquiries as to individuals necessitates a selection of cases, and information of a reliable nature can only be obtained on every point from few persons. In the case of new-comers the date of arrival in a country and the subsequent movements, with approximate dates, are usually to be trusted. The date of the first attack of malaria can generally be obtained. Sufficient information about the attack, such as the character and duration of the "fever"; the effect of quinine, and absence of any other cause of pyrexia, such as septic infection or pneumonia, must be ascertained to render it probable that the attack was malarial.

Any form of indisposition in the older residents is

so frequently called malaria that less reliance is to be placed on these than on the history of the first attack. In malaria it must always be remembered that relapses are so common that a second *attack*, even at an interval of several months, does not prove a second *infection*.

The Liability to Relapses is more difficult to determine, but with a fair number of individuals it can be ascertained, and great individual variations will be found. In newcomers three weeks to a month is a common interval, whilst in others the period may be as long as four or six months. In this connection careful enquiries as to the habits as regards quinine are of great importance, as if quinine is taken constantly, even in small doses, the relapse is often postponed till the quinine is discontinued.

Increase in the Interval between Relapses.—Any observations as to increase in the interval between the relapses with increased length of residence or diminution in the severity of the attacks may indicate that a degree of immunity has been acquired, and the length of residence required for this, though shorter in all in the more malarial districts, is to a great extent an individual peculiarity.

As regards the population in general, it is essential that the actual numbers of the different races represented be known before any use can be made of totals, such as number of deaths, admissions to hospital, &c. This warning may appear superfluous, but it is not. In published reports one of the commonest errors is to speak of a disease as being more or less prevalent in a district on the ground of the number of cases seen, not as it should be, on the proportion of the susceptible population attacked.

It is in connection with blackwater fever and yellow fever that such errors are most common.

Age Incidence.—Personal observations should be made

on unselected cases and the number of cases examined mentioned in the table, with the percentages. Ages cannot be ascertained with certainty, especially in countries where the differences in season are not very marked. With children age has to be estimated from the size, teeth* and development. In adults knowledge of local history and notable events, the dates of which can be fixed, are of considerable value. Age periods of five years are usually taken, but it is of the utmost importance in malarial investigation to subdivide the first quinquennial period and further subdivide the first year into quarters. The first quarter should be subdivided into months. Malaria is rare till the end of the first month. As an age period the first ten years should never be taken as a whole, as such different results are obtained in a village, or in a series of observations, if a large proportion are, say, under four, or only a small proportion. Conclusions drawn from the incidence of

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* Ages at which teeth are cut in *Europeans*. The differences in native races have not yet been worked out. Table kindly supplied to me by Mr. K. W. Goadby.

TEMPORARY DENTITION.

Central incisors	5th to 8th month.
Lateral incisors	7th to 10th ,,
First molars	12th to 14th ,,
Canines	14th to 20th ,,
Second molars	20th to 30th ,,

PERMANENT DENTITION.

	Upper Jaw.	Lower Jaw
Central incisors	7·7 years ...	7 years.
Lateral incisors	8 ,, ...	8 ,,
Canines	11 ,, ...	10 ,,
Premolar I.	10 ,, ...	10 ,,
Premolar II.	11 ,, ...	11 ,,
Molar I.	6½ ,, ...	7 ,,
Molar II.	12 ,, ...	12 ,,
Molar III.	24 ,, ...	24 ,,

malaria in the first ten years of life, taken as a whole, are often misleading.

Mortality is best estimated at the rate of so many deaths per 1,000 per year, as then the results can be compared. If dealing with short periods, as, for instance, one week, the death-rate would be the proportion of deaths per 1,000, of the population in that period, multiplied by 52. If a long period, say ten years, is taken the death-rate would then be represented by the number of deaths per 1,000 divided by 10.

The factors necessary are the number of persons of the required class alive at the commencement of the period, the number of deaths of this class who died from the disease which it is desired to investigate in the period, and the length of the period.

Case Mortality is the percentage representing the proportion of cases terminating fatally. The number of cases of the disease and the number of deaths from the disease are the only two factors requisite. If it is desired to compare the "case mortality" in different years or other periods of time, cases occurring in those periods only must be included. In malaria untreated and treated cases must be considered separately, and the treatment mentioned as the case mortality is so much reduced by effective treatment.

Remote or Indirect Mortality is the mortality due to remote complications, visceral changes and increased liability to other diseases, or to the tendency which malaria appears to have to aggravate some diseases. Our knowledge of this branch of the subject is most inaccurate and requires complete revision.

The effect on general health varies greatly in different conditions, and under circumstances little understood. Splenic enlargement, anæmia and diminished rate of growth are the most definite. Susceptibility to tuberculosis appears to be induced by chronic malaria in

countries where tuberculosis is prevalent. The effect on the general health, apart from the actual attacks, whether mild or pernicious, varies according to race.

Period of natural *incubation* and its variations can be determined from the histories of patients, and then must be limited either to first attacks or to other attacks in which a long interval has elapsed. The most common history given is of some immediate antecedent. Exposure to chill, constipation, change of residence, particularly from a warmer to a cooler place, and even cessation of travelling, are given as the causes of the attack. These causes are not to be taken as those of the infection, though they may determine or accelerate the manifestation of the disease.

The time of actual onset of symptoms can usually be told with certainty, but the time of infection is difficult to determine. The frequency with which travelling in one form or other enters into the causation is usually to be ascribed to passing through a highly malarial district, or even to spending some hours in a house where infected mosquitoes are to be found. With a sufficient number of cases it is sometimes easy, as in the case of a steamer, or in persons travelling over known routes, to fix on the date of infection as the date on which a halt was made at a notoriously malarial place. Such cases show the wide limits of the period of natural incubation, often longer than those which have been determined experimentally by feeding infected mosquitoes on susceptible persons.

The *evidence of immunity* is to be considered under two heads: (1) Age incidence of the disease in natives and cessation of attacks with advancing years. (2) In newcomers the residential period during which attacks occur and any evidence, by the diminishing frequency or severity of attacks, that some immunity is acquired.

With malaria it is important to consider whether

there are periods in which from climatic conditions infections do not take place. In the case of individuals, if there are periods during which they are not resident in places where malarial infection is possible. Immunity is destroyed or diminished by such periods, so that if they are long immunity is not acquired at so early a period, or at all. There is evidence that immunity is not of long duration in malaria, but more exact observations are required on this point.

In any consideration of immunity the liability to infection—endemic index—must be taken into account, as with a low endemic index individuals only, not a class, will acquire immunity.

(3) *As regards Place.*—In considering any place it is important to bear in mind that malaria is a local disease, and that even in houses close together it appears, that one will be more malarial than another. Still more so are different quarters of the same town or district, and the localities where the disease is most prevalent vary from year to year. These differences and the causation of the variation in the differences require local investigation in all cases.

Seasonal variation may act in two ways, first by rendering the conditions more favourable for the multiplication of *Anopheles*, and secondly by presenting conditions more favourable for the development of the malaria parasites in the mosquitoes. Rainfall, both the amount and distribution, *i.e.*, whether in frequent light showers with short intervals, or heavy downpours with long intervals, is of great importance, and so is the level of the subsoil water, which may be more affected by distant rain than by the local rainfall. A high temperature within certain limits causes more rapid breeding of mosquitoes, causes them to require food more frequently, and is favourable to the rapid development of the malaria

parasites, and so in all these ways will favour the spread of malaria.

The *species of Anopheles* present and of those most numerous in the district should be determined, and these species of mosquitoes should be tested as to the readiness with which they may become infected by the malaria parasite. Different species vary greatly, and even with the same species infection seems to occur with varying difficulty under different circumstances. As a rule mosquitoes reared from larvæ are not as easily infected as those caught in the adult stage. The possible or known circumstances affecting the development of the parasites are the temperature, the age of the mosquito, whether impregnated or not, and the nature of the food previously taken by the mosquito, and probably other conditions which all require local investigation.

Endemic Index.—A numerical estimate of the liability to malarial infection is an important factor to determine. The number of malarial attacks, or of hospital admissions for malaria, from amongst a known number of persons, is of little value, even if the diagnosis is confirmed in every case by blood examination, as these admissions will include recurrences and relapses which will vitiate the figures, *e.g.*, a man infected once with malaria may have a dozen attacks of malaria as a result of this single infection in a year, or he may have only one. In the first instance he would appear in returns as 12, in the second as 1, though in both instances for our purpose they should be represented as 1.

If first attacks only are included this difficulty does not occur, and first attacks have the further advantage of being usually severe, and in persons who have not yet acquired the habit of treating themselves and who therefore come under medical observation.

For an estimate of the liability to infection, or *endemic*

index, by this method, the factors to ascertain are the dates of first attacks of malaria occurring during the course of the observations verified by blood examinations or in other ways, effect of quinine, &c., and the length of residence previous to the attack, and the number of newcomers during the period who have escaped infection. As a separate estimate a statement by as large a proportion as possible of the resident population as to the length of time they had each resided in the country before their first attack of malaria. These figures usually lead to much the same result. Reliance has to be placed on histories only, and errors may occur, though each factor is one which most of the residents are capable of observing.

By this method the length of residence in weeks or months that is ordinarily required for an attack of malaria is determined. The period of incubation we know varies, but is commonly from ten days to three weeks, and this period should be subtracted from the length of residence required for an attack of malaria to obtain the period of residence required for an invasion.

Where bodies of men are working together and are under medical observation, as in regiments, gangs of workmen, &c., this method is, I believe, the best and simplest, and includes no sources of error that are not common to other methods.

In such an estimate all persons who were born and have lived in malarial countries for prolonged periods should be excluded; also those who have contracted malaria in other malarial countries. For these exclusions there are two reasons, (1) to avoid including relapses, and (2) to avoid including persons who may be immune.

A somewhat similar method is to determine the proportion of untreated natives who harbour the parasites of malaria. In this method the ages must be known and unselected children, including those apparently in

good health, must be included, and children should form a large proportion of the cases.

This method has been extensively used and an arbitrary standard, ten years, has been selected ; the proportion of children under 10 years of age with malarial parasites is then taken as the index.

A more satisfactory method is to determine the proportion at different ages. Thus in one district, whilst 86 per cent. of the children under 2 years of age were infected with parasites of malaria, only 28 per cent. of those from 5 to 10 harboured them. If, therefore, in such a place most of the examinations were made in young children, a much higher index would be obtained than if most of the children were over 5.

In many of the determinations no further information than "children under 10 years of age" has been given, and in some of them the number of children examined is very small.

It is not very easy in some places to get a sufficient number of children for examination, but with patience it can generally be done. As these cases are untreated, many of them, if not most, will have had the parasites for considerable periods, and therefore the figures only indicate antecedent, perhaps remote, *infection*. If young children were examined monthly till parasites were found the liability to infection under native conditions would be determined more accurately. In making any series of blood examinations the time selected should be during a period of settled weather. If examinations are made during a change, particularly from hot to cold, the parasites will be more easily found, as the effect of chill is to favour the development of the parasites. Examinations made at such times will therefore show a higher index than those made in settled weather.

The Spleen Test, or the proportion of persons with enlarged spleens, is useful if age and race are taken into

account. It is of more value amongst negroes than amongst other races, as the negro spleen does not continue to enlarge after immunity has been acquired in the same way that the spleens of many individuals of other races do. The test can be used easily as there is nothing in the examination to excite alarm or frighten the children, and can be made more quickly than any other examination.

It indicates only antecedent, probably remote, infection, and is less certain proof of antecedent infection than the presence of parasites.

A large proportion of enlarged spleens between 2 and 5 years of age is an indication of a high endemic index. If the presence of malaria in a district is proved, the absence of enlarged spleens in negro adults, or a low proportion between 10 and 15, is equally a proof of a *high* endemic index, whilst if the proportion of enlarged spleens in adult negroes is appreciable or large in those between 10 and 15 the endemic index is *low*.*

The determinations obtained by the spleen test are less liable to be influenced by meteorological conditions than the test by blood examinations, are easier, and can be made in a larger number of cases, but otherwise are less accurate, as the conditions that lead to splenic enlargement after malarial infection vary and are not thoroughly understood, and splenic enlargement may be due to other causes.

Another proposed method of estimation of the index is by determining the proportion of the *Anopheles* that are found to be infected with the parasites of malaria. For this method to be of value the mosquitoes must be selected from different houses and places in equal proportion, as it will be found that there are great variations

* With no other race but the Negro can such conclusions be drawn.

in this proportion in adjoining houses and at different times. One good "crescent case" will infect almost every *Anopheles* of certain species that bites the patient, whilst only a small proportion of those that bite the more numerous poor crescent cases will be infected. *Anopheles* in European houses are rarely found to be infected, whilst in an overcrowded native house, where there is no protection of the inmates from mosquitoes, a large proportion of infected *Anopheles* may be found.

The *proportion* of infected mosquitoes is not the real test so much as the *number* of infected mosquitoes, so that in these estimates the number of *Anopheles* that bite a man per hour is also required.

A high endemic index, as determined by the other methods, will be found in places where *Anopheles* are very numerous, even when the proportion infected is very small.

It must always be remembered that a place with a large number of *Anopheles* of species known to be good carriers of the parasite, even if free from malaria at any one time, has the potentialities of a high "endemic index" if the place is inhabited by newcomers or other persons susceptible to malarial infection. This is the reason why railway and engineering works are so often attended with outbreaks of malaria, even when conducted in places that previously were not considered to be very malarious. *Anopheles* are present and perhaps numerous; in any case many new breeding-places are formed during excavations and the mosquitoes become numerous. Gangs of workmen are crowded together in temporary huts and they are not protected from mosquito bites. The workmen will include susceptible newcomers, and frequently some persons harbouring parasites. A single good "crescent case," often a man with no symptoms of malaria, will infect a number of mosquitoes, and in the course of some ten days or so

these mosquitoes will infect any susceptible persons who sleep in the hut.

The earliest numerical estimate was arrived at by determining the proportion of persons at different ages whose organs contained malaria pigment. This method can only be adopted under circumstances where *post-mortem* examinations can be obtained in both children and adults. The results indicate antecedent malarial infection. The method, though fairly good, is only of limited value, as the large number of *post-mortem* examinations required can only be obtained in few places.

These, then, are the main methods for the determination of the endemic index :—

(1) By determining the length of residence required to render malarial infection probable in susceptible newcomers.

(2) The ages at which the largest proportion of natives harbour the parasites of malaria.

(3) Ages at which splenic enlargement is common.

(4) Percentage of persons dying from all causes with malarial pigmentation of the organs.

(5) Number of infected *Anopheles*.

Other warnings, though too complicated by other factors to be used numerically, are a high infantile death-rate amongst the natives, particularly a high death-rate from convulsions in infants over six months; a high European death-rate; and, I am inclined to add, occurrence of blackwater fever.

Graphic representations in the form of "charts" are useful as indicating the main results of any enquiry, as they are easier to follow with the eye than columns of figures or rows of statistics.

The essential of a good chart is that it should be capable of translation back into figures, *i.e.*, a chart should be such that it can be read.

The principle of charting on a plane surface in two

dimensions is that the horizontal line represents one factor, usually time or periods of time, whilst the vertical represents the other factor.

Each of the factors should be represented according to scale in order that it can be read, and this point is often overlooked, even in the familiar temperature charts, in that whilst the height of the temperature is recorded correctly in the vertical columns, the distances measured horizontally between the points representing the different observations are equally spaced, so as to look neat, whilst the real intervals of time are irregular, *e.g.*, temperatures are taken at 2, 10, 12, 3, on a four-hour chart, and should be so recorded that the distances measured on the horizontal line are unequal in the proper proportion.

In that case the chart can be correctly translated back into figures, otherwise, if represented as equi-distant the translation would read 4, 8, 12, 4.

The limit to the translation of the chart is the scale of the chart; where the intervals allowed for time are small, translation can only be approximate.

In blood charts it is usual to represent the heights as percentages of normal, as real figures would require such enormous charts if it were desired to represent graphically on the same scale both red and white corpuscles. In such cases it is better to keep to the figures or to use different vertical scales for the more numerous and more scanty elements. It must be clearly indicated for each what the scale represents if this be done.

Too many lines on one chart are difficult to follow, and the only cases in which it is advisable to have two or more lines is when it is desired to compare two or more results.

The different methods of determining the endemic index of malaria are conveniently rendered graphically and serve as illustrations of the method. Chart I. is compiled from an official report of W. H. G. H. Best, of

the Lagos Medical Service, and formerly of the London School of Tropical Medicine, which is almost the only report published that gives sufficient details for the determination of the age incidence.

CHART I.

——— Negroes (Native Africans).—Hausa and Yomba Children, 320; Hausa Adults, 100. Compiled from Official Report, Lagos, of W. H. G. H. Best.

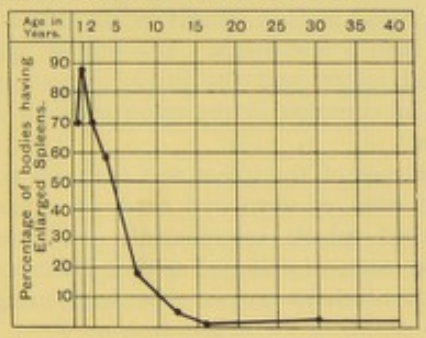
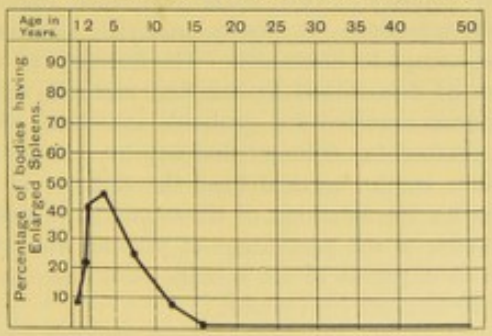


CHART II.

——— Negroes (Native Africans), Central Africa.—714 Native Children under 15, and numerous Adults.



No cases are given under 3 months of age, and those under 6 months are very few. The chart shows clearly that under the conditions of a native life a large propor-

tion of children are infected in less than six months, and practically all in less than a year, whilst the number of infected children after five years is so small that the majority must have acquired immunity.

Chart II. shows the age incidence of enlarged spleens in Central Africa, and on chart III. are shown the same cases subdivided into two widely different groups; in the one district Europeans often pass their first year without getting malaria, whilst in the other few escape for more than a few weeks. The earliest age incidence of enlarged spleen and the earlier period at which it ceases to be common in the more malarial district are well shown.

The weakness of the spleen test is that a considerable proportion even of untreated cases of malaria do not show marked splenic enlargement, and it is probable that 68 per cent. of enlarged spleens indicates universal infection as much as 90 per cent. harbouring parasites would do.

Chart IV. indicates the proportion of persons at different ages with malarial pigment in the spleen in a moderately malarial country; two years' exposure required for probable infection. It shows malarial infection later and less complete immunity. The liability to malarial infection as determined by the first method, the length of residence required for probable infection, would be simply charted for different districts by representing for each place in the vertical lines the number of months requisite for the infection of three-fourths of susceptible new-comers, or the percentage of persons who would be infected in a period of six months or a year, as is considered to be most convenient.

In some charts the distances on the horizontal line have no meaning, and it is simply for convenience that the horizontal spacings are made, a series of vertical columns packed together or widely and irregularly

separated would have the same meaning but cause confusion.

The convenience of such charts is that various points can be indicated on the same chart and compared.

CHART III.

——— Negroes (Native Africans).— In a most Malarial District in Central Africa. Residence Required for Probable Infection with Malaria, under Six Weeks.

------ Native African.—In less Malarial District. Residence for One Year does not render Infection Certain.

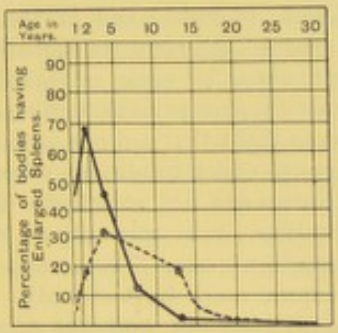
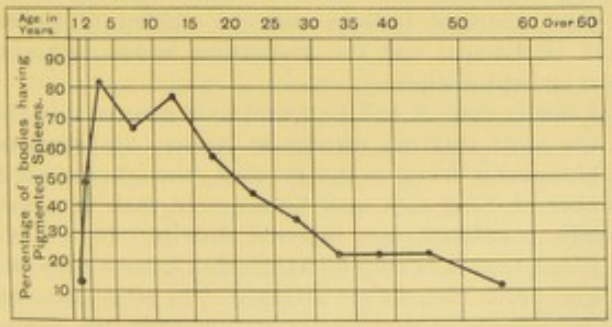


CHART IV.

—— — Negroes (Native Africans).—Compiled from Post-mortem Examinations in British Guiana.



The line commences at one month, no pigmentation being found earlier. The next point is under six months.

Many statistics before charting require correction. Of these corrections some are obvious and easily made. The number of cases is useless unless the total number of the population that are susceptible to the disease is also known. In dealing with inhabitants of different races any difference in the susceptibility has to be noted and allowed for.

Blackwater fever is a good illustration in point, and so many erroneous statements are made in connection with it that it well serves as an illustration. It occurs in Tropical Africa, in India, and in the West Indies, amongst other places. All races are probably susceptible, though this is doubted by some as regards some negro races: in any case the susceptibility varies; and the negro susceptibility is so slight that not one in many thousands will get the disease under conditions where some 8 per cent. of the Europeans are attacked. The Indian is certainly susceptible, but only about one-fourth as susceptible as the European. These variations in racial susceptibility require much further study.

We must know, therefore, both the number of Europeans and the number of cases that occur amongst them in each district before we can compare the prevalence of the disease in each district. Similarly the number of cases amongst Indians and the number of Indians must be known, and the proportion in the two races must be kept distinct.

If this is done it will be found as a general rule that in the most malarial district in Africa the prevalence of blackwater fever is the greatest, though the actual number of cases seen may be no more than in a more thickly populated, less malarial district. It will also be found that it is only in Africa that a *large proportion* of susceptible persons are attacked. In other countries, where perhaps as many *cases* may be seen, the number of susceptible persons is much larger.

Another correction, an important one, has to be made. Unfortunately the amount of the correction is dependent on a variable factor—the period of incubation of the disease. More cases of blackwater fever probably occur in England than in any one small district in Africa, but these cases are all in people who have returned from Africa and acquired the infection there. In these cases there is no doubt that the infection should be attributed to the part of Africa from which they came. Here the matter is easy, but in Africa itself it is so often found that persons develop the disease who have been travelling, that it is a matter of great difficulty to attribute the disease to the correct place of origin. In many cases the place where the disease develops is certainly not the place where it was acquired. The correction to be applied here is essential, but can only be an approximate and arbitrary one. Personally I prefer to take the place of residence a fortnight before the attack as the more probable place to be implicated in a large proportion of the cases.

Charting is often useful to represent the secretion or excretion rates either of definite substances, such as urea, or the volume of a mixed fluid, such as urine. Here times are represented by the distance measured horizontally, and amounts, weights, or volumes by the height measured vertically.

The only difficulty is that however it may be secreted urine as well as other fluids are only passed at intervals, and it is the rate at which urine is being formed, not that at which it is being passed, that is of importance. The only available method is to divide the number of ounces of urine passed, or, if necessary, drawn off by catheter, by the intervals measured in hours between the successive micturitions; the result will give the average rate per hour, assuming that the bladder is equally empty after each micturition.

Such charts are of a special value in diseases like blackwater fever, in which there is a tendency to suppression of urine, and may indicate the periods of greatest danger.

The geographical and topographical distribution of disease, of parasites and of certain insects is of considerable importance. Maps should be drawn to the required scale and the places where an examination shows that the condition to be charted is present marked with a plus +, and where absent negative, —. Places where no observations have been made should be clearly indicated, for if, as is sometimes done, they are represented as negative, most misleading conclusions as to the distribution may be drawn.

It is usual to represent the incidence of a condition by shading, and the depth of the shading indicates also the prevalence of the condition.

In determining the incidence of a disease in a town or village a plan must be drawn up and the houses or groups of houses infected indicated as above.

Extraneous conditions, such as wells, streams, or other sources of water, must be shown, and when dealing with a question such as malaria, known to be carried in a certain way, other conditions, favouring the prevalence of such carriers as *Anopheles*, must also be indicated.

These maps and charts enable the conditions to be quickly understood, and are therefore of considerable value if accurate and carefully drawn up.

With a little ingenuity almost anything can be represented in a graphic manner, or charted. The value of a chart is the ease with which relations are shown and with which conclusions can be deduced. They show no more and prove no more than the figures or facts they represent, but by many are more easily followed. If, therefore, a chart does not represent matters more clearly than the figures the chart is useless.

APPENDIX.

Instruments and Reagents.

Microscope, with two eye-pieces, 2 and 4; three objectives, $\frac{2}{3}$ in., $\frac{1}{6}$ in., and $\frac{1}{12}$ in. oil immersion lens; substage condenser, and iris diaphragm and mechanical stage, micrometer eye-piece with scales or with squares, micromillimetre scale, camera lucida.

Watchmaker's glass.

Portable microscope.

Direct vision spectroscope

Slides, No. 2 quality.

Cover-glasses, No. 1 quality, to be packed in oil.

Needles in handles Cork felt. Entomological pins, No. 20. Forceps. Cornet's forceps. Mounted platinum wires.

Test tubes, thick and best quality. Durham's tubes. Watch glasses. Petri dishes. Photographic trays, half- and full-plate. Erlenmeyer's flasks. Funnels. Glass tubing. Glass rod. Beakers. Burette, 50 cc. Evaporating dishes and copper dish for boiling slides. Spirit Bunsen. Prima's kerosine lamp.

Glass measures, 500 cc., 100 cc. and 10 cc.

Scales. Gramme weights.

Paraffin oven. Paraffin moulding dish and blocks.

Microtome.

Steam steriliser. Hot-air steriliser and incubator. Iron enamelled jugs.

Mounting and Imbedding Reagents.—Alcohol, cotton-wool, methylated spirits, oil of cloves, xylol, Canada balsam, glycerine, Farrant's solution, glycerine jelly, ether, chloroform celloidin, paraffin wax, Hollis' glue, or shellac.

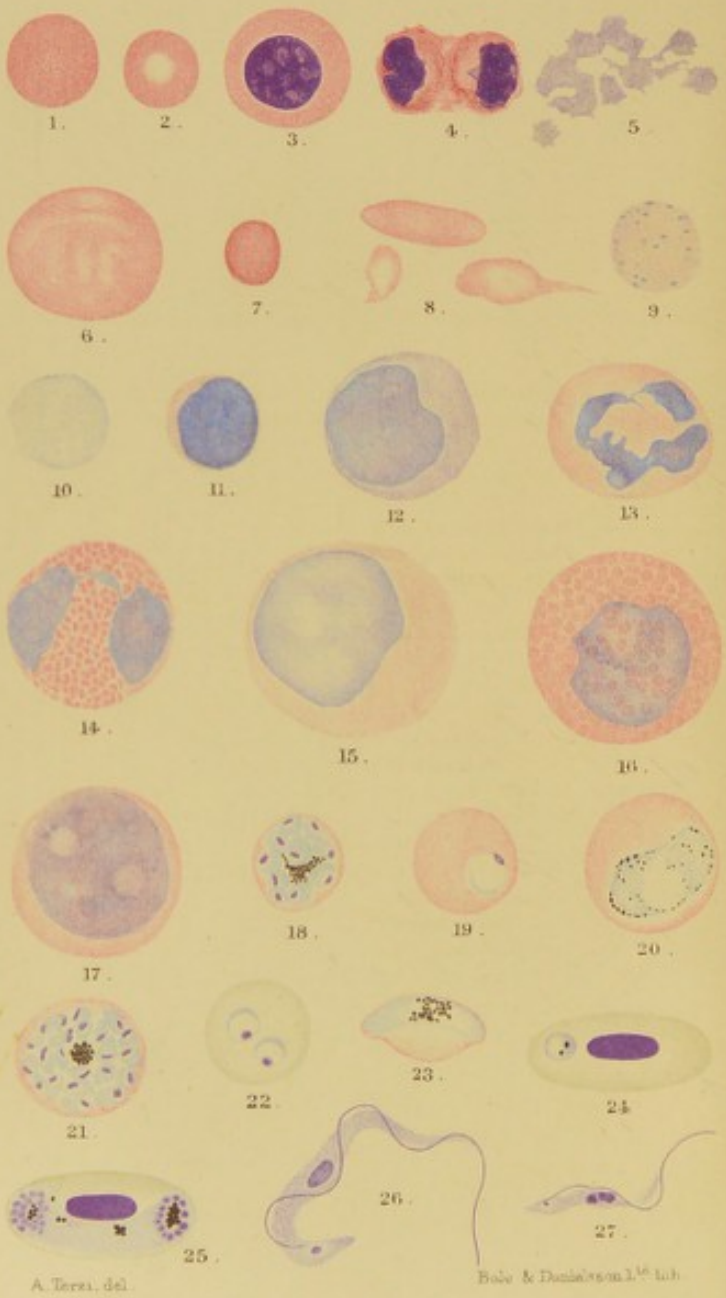
Stains.—Hæmatoxylin crystals, hæmatein, methylene-blue,

(Höchst's pure medicinal), thionin, gentian violet, fuchsin, carmine, picrocarmine, toluidine blue, night blue, bismarck brown, methyl violet, eosine, both soluble in alcohol and soluble in water. *Grübler's stains are the best.*

Other Reagents. — Acids: Hydrochloric, nitric, sulphuric, picric, osmic, tannic, carbolic (pure), gallic. Agar-agar, alum, ammonia, alcohol, methyl alcohol (pure for analysis), borax, iodine, filter paper, filter paper (Chardin), formalin, gelatine, glucose, lactose, lithium carbonate, lysol, mercuric chloride, naphthaline, peptone, platinum chloride, potassium ferrocyanide, potassium iodide, potassium bichromate, phenolphthalein, sodium carbonate, sodium citrate, sodium hydrate, sodium sulphate, sodium taurocholate.

, thionin, gentian violet, fuchsin,
luidine blue, night blue, bismarck
ine, both soluble in alcohol and
stains are the best.
: Hydrochloric, nitric, sulphuric,
ic (pure), gallic. Agar-agar, alum,
alcohol (pure for analysis), borax,
aper (Chardin), formalin, gelatine,
rbonate, lysol, mercuric chloride,
tinum chloride, potassium ferro-
, potassium bichromate, phenol-
e, sodium citrate, sodium hybrate,
aurocholate.

Plate 1.



A. Terzi, del.

Bale & Danielsson, Lith.

PLATE I.

STAINED WITH HÆMATOXYLIN OR EOSINE AND HÆMATOXYLIN.

Figs.

1, 2. Normal variations in red blood corpuscles.
3, 4. Nucleated red blood corpuscles.
5. Blood plates.
6, 7. Abnormal variation in size and colour.
8. Abnormal shapes, poikilocytes.
9. Basophilic granules.
10. Polychromatic red corpuscle.
11. Lymphocyte.
12. Large mononuclear leucocyte.
13. Polymorphonuclear leucocyte.
14. Eosinophile leucocyte.
15, 16, 17. Myelocytes.
18. Quartan rosette.
19. Young tertian parasites.
20. Half-grown tertian parasite.
21. Rosette benign tertian.
22. Malignant tertian (sub-tertian), ring form. Double infection of corpuscles.
23. Crescent. Gamete malignant tertian (sub-tertian).
24. Young halteridium.
25. Sporulating halteridium.
26. Trypanosome of fish.
27. *Trypanosoma Brucei* (tsetse fly disease, nagana).

PLATE II.

MALIGNANT TERTIAN PARASITES (SUB-TERTIAN) STAINED WITH CARBOL THIONIN.

Figs.

1. Young form, rings.
2. Half-grown parasite.
3. Full-grown parasite.
4. Sporulating parasite.

3a, 4a. Are full and sporulating parasites, as seen in sections of organs shrunk by the spirit and other processes.

5, 6, 7, 8. Development of the gametes of malignant tertian.

9, 10. Benign tertian parasites, half-grown and sporulating.

11, 12. Quartan parasites, half-grown and sporulating.

13 to 16. Sporozoa of cattle and horses.

Embryos of Filaria Stained with Hæmatoxylin.

17. *F. nocturna.*
18. *F. perstans.*
19. *F. Demarquaii.*

Plate II.


1.

2.

3.

3a.

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4a.


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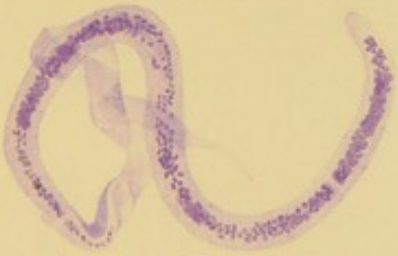
16.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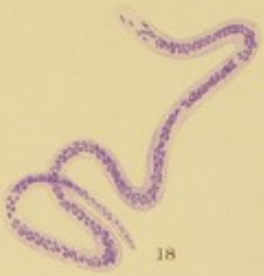
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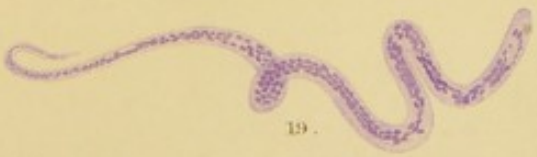
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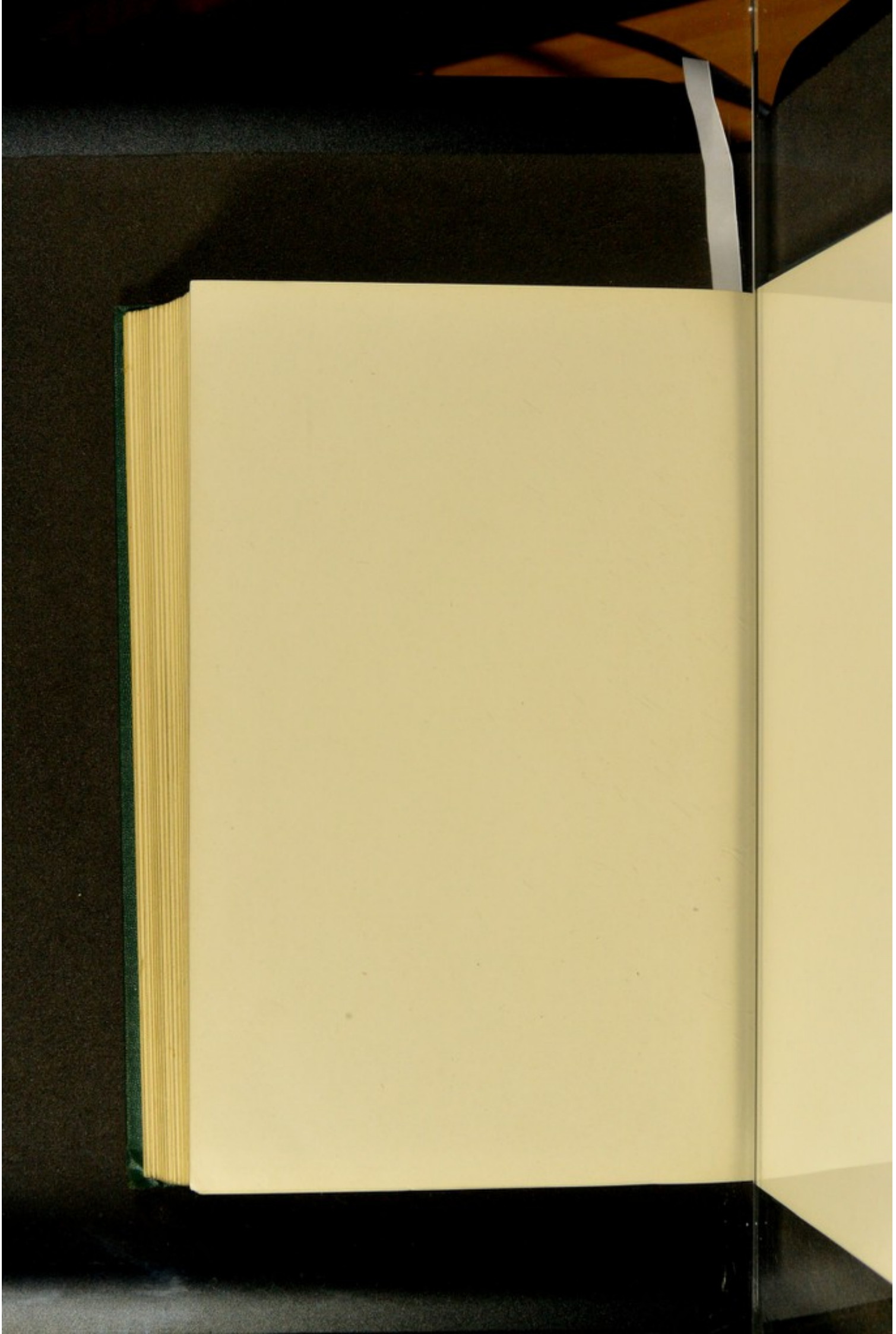
A. Terry, del. Dale & Donnelson, Lith. Lith.



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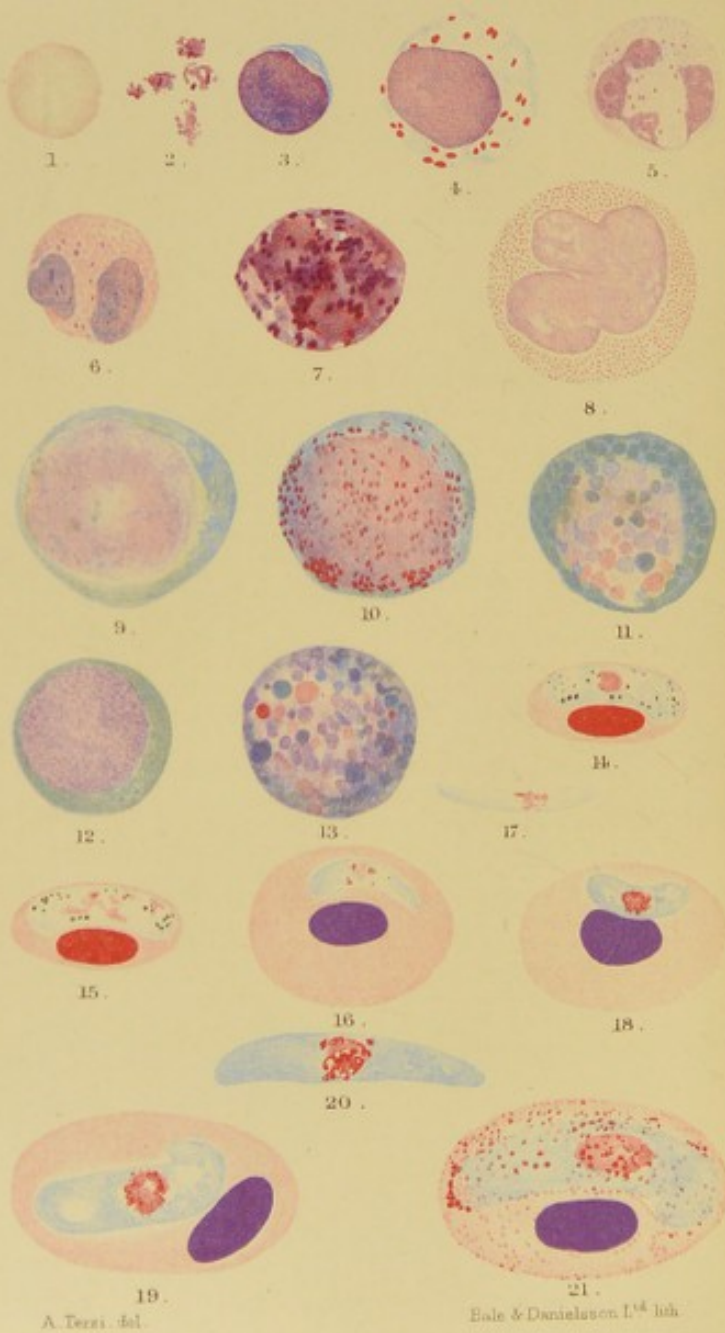


A. Terzi del.

Hale & Danielsson Lith.

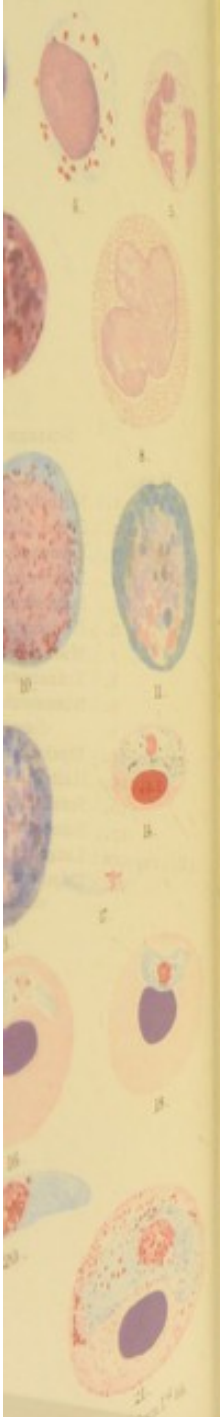


PLATE III.

STAINED WITH LEISHMAN'S STAIN.

Figs.

1. Normal red corpuscle.
2. Blood plates.
3. Lymphocyte.
4. Large mononuclear leucocyte.
5. Polymorphonuclear leucocyte.
6. Eosinophile leucocyte.
7. Mast cell.
8. Transitional form.
9. Abnormal mononuclear cell found in certain diseases, including *trypanosomiasis*.

10 to 13. Myelocytes showing various types of granules.

14, 15. Halteridium.

16. Small drepanidium in red corpuscle.
17. Same drepanidium in plasma.

18, 19, 20. Large drepanidium in various stages.

21. Degeneration of red corpuscle caused by drepanidium (Schüffner's dots).

PLATE IV.

STAINED WITH LEISHMAN'S STAIN.

Figs.

1 to 5. Stages of benign tertian parasite.

6. Gamete benign tertian.

7, 8, 9. Characteristic degeneration of red corpuscles containing benign tertian parasites (Schüffner's dots).

10 to 15. Stages of quartan parasite.

16, 17. Stages of malignant tertian (sub-tertian) which are seen in peripheral blood.

18. Male gamete, malignant tertian (sub-tertian).

19. Female gamete, malignant tertian (sub-tertian).

20. Double infection with malignant tertian (sub-tertian) parasites of a red corpuscle; basophilic granules in red corpuscle.

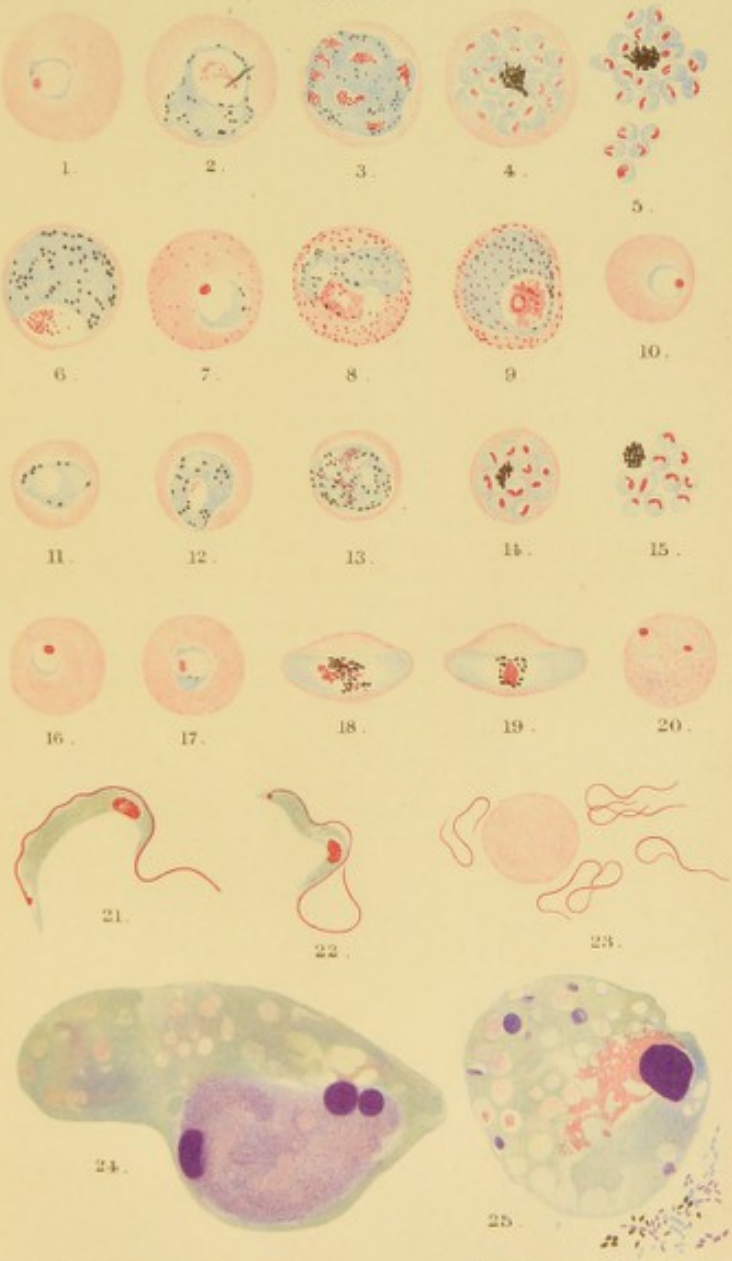
21. *Trypanosoma Lewisi* (rat).

22. *Trypanosoma hominis* (Congo).

23. Spirillum of relapsing fever (stained with carbol fuchsin).

24, 25. *Amœba coli.*

Plate IV.



A. Terzi del

Bale & Danielsson, Ltd. lith.

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