The Structure of Trypanosoma lewisi in Relation to Microscopical Technique.

By

E. A. Minchin,

Professor of Protozoology in the University of London.

With Plates 21, 22, and 23.

Introduction.

Much has been written of late years about the minute structure of trypanosomes, and also about the merits or demerits of the various methods in vogue for preparing them for examination with the microscope, that is to say, the technique of fixing, staining, and preserving these tiny creatures. Since our knowledge of the structure of trypanosomes is based almost entirely on the results of a number of complicated chemical and physical processes practised on a very delicate protoplasmic body, it is clear that a knowledge of the effects produced by these processes on the organisms are most important in interpreting the microscopic image finally obtained, in order to estimate, in any given case, how far the trypanosome may have undergone deformation or change as the result of the treatment it has gone through. In a perfect state of scientific knowledge it would, no doubt, be possible to deduce exactly such results from the known action of the reagents employed upon the protoplasmic body, but in the present condition of our knowledge it is only possible to arrive empirically at an approximate estimate of the effects of technique by comparing carefully the results yielded by it.

In order to eliminate as much as possible disturbing factors

due to variability in the objects themselves, I have made use in these researches of the common trypanosome of the rat, since it can be obtained in a practically monomorphic condition and does not exhibit the remarkable polymorphism shown, for example, by the trypanosome of sleeping sickness. Moreover, after about the tenth day of infection all multiplication ceases, and though individuals may be found occasionally with two or even three trophonuclei, I am convinced that this condition has nothing to do with division, as has sometimes been supposed, but is to be regarded as an abnormality. Hence in Trypanosoma lewisi, after the multiplication-period is over, the variability in size and structure is reduced to a minimum, and the details of cytological structure are not complicated by changes due to processes of fission or multiplication.

This memoir is divided, therefore, into two parts. In the first part I shall deal with the subject from the point of view of the technique, and discuss the results obtained by different methods of fixing, staining, etc., under their respective headings. In the second part I shall discuss the structure of the trypanosome itself, taking its various parts in order. In dealing with two subjects which, though distinct, necessarily overlap to a certain extent, it is very difficult to avoid repetition, and though I shall try to steer clear of this defect in exposition as much as possible, I must crave indulgence in those parts of my subject where repetition is an alternative to omission of necessary statements.

Part I.—Technique.

The subject of modern microscopical technique is an absolutely inexhaustible study; no one in a human life-time could claim to have said the last word upon it. As I write, all sorts of methods that I have not tried, or variations upon methods that I have tried, present themselves to my mind. It becomes necessary in a research of this kind to set a term to one's work, and to rest content at a certain period with what one has achieved, however incomplete it

may appear to oneself or to others. Circumstances, into which I need not enter, oblige me to break off at the point I have reached, and to bring forward my results, such as they are, without undertaking further investigation.

The object of microscopic technique when applied to an organism such as a trypanosome is to produce a microscopic image which shall represent, so far as possible, the form and minute structure which we believe the organism to possess actually in the living state, true in all details, exact to a definite known scale of magnification, and coloured artificially so as to assist in rendering visible the different parts of the organism. A method which would have this ideal result would be a perfect method; but unfortunately no such method is known to exist, since all technique deforms or falsifies the form or structure of the organism to a greater or less extent. We are therefore confronted at the start with the difficulty, that since we can only arrive at a conception of the true and actual structure of the objects by the use of methods of technique, we are obliged to estimate the deviations from the truth, produced by technique, solely by a comparison of results which are themselves one and all defective. The only conceivable method by which the true form and structure of Trypanosoma lewisi could be recorded would be by a photograph of it in the living state; but I know of no method by which a snapshot can be taken of a minute and transparent organism, in a state of incessant and active movement, at a magnification of 2000 or 3000 diameters.

It was necessary, therefore, to find at the outset some method of killing and preserving the trypanosomes with the least possible deformation or alteration, in order to serve as a standard of comparison for other methods. It has always been my experience, and I think that of others, that in the case of Protozoa of larger size, such as Ciliata, Amœbæ, etc., the most life-like preparations can be obtained by simply exposing suddenly the living organisms, moving freely in a small drop of the medium they inhabit, to the vapour of strong osmic acid. The organisms are then examined without further

treatment, except that the preparation is sealed up to avoid evaporation of the fluid medium, and in this way it is possible to obtain very perfect temporary preparations, which can be kept unaltered for at least some months. I made use, therefore, of this method for trypanosomes, but on account of the medium, blood, in which they live, special precautions were necessary in the application of it.

My first method was to take an ordinary microscope slide with a small depression hollowed out in the centre, and to cement on to this a ground-glass ring, surrounding the hollow. The upper edge of the ring was then painted with vaseline, and a drop or two of osmic acid solution (4 per cent.) placed in the hollow of the slide. Now a coverslip was taken and a drop of fresh blood placed in the middle of it and spread out with a clean glass rod, not, however, smeared out into a thin layer. It was then placed with the blood downwards on the ground-glass ring over the osmic acid, and the coverslip pressed down all round on the vaseline. All this manipulation was done with the greatest possible rapidity, in order to avoid evaporation of the blood as much as possible. Thus the blood in a hanging drop is exposed to the vapour of strong osmic acid in an air-tight chamber; it is, of course, important that the blood should not come into contact with the osmic acid solution. It is now possible to examine the trypanosomes without further treatment; but owing to the thickness of the preparation, difficulties arise in the illumination of the object, since it is not possible to focus the substage condenser properly for the use of the highest powers. I was able, however, to draw the trypanosomes in outline, at a magnification of 3000 (figs. 1, 2), but could not make out minute details. In order to do this a further manipulation was necessary.

After the coverslip with the blood had been exposed to the osmic vapour for a certain time, it was picked off the glass ring and at once placed with the blood downwards on an ordinary clean side. From the glass ring the coverslip takes with it a ring of vaseline, which when pressed down on the

slide forms an air-tight cell for the enclosed blood. To ensure against the evaporation the coverslip is further luted all round the edge.[1] The blood is thus removed from the osmic chamber with no other alteration than the amount of evaporation which it suffers during the rapid passage through the air from the glass ring to the clean slide. Preparations made in this way keep without perceptible alteration for months; the trypanosomes can be examined with any power of the microscope and drawn to any scale; it is possible to make out all details of structure in them and even some which cannot be made out in other ways (figs. 3–8). The trypanosomes have undergone no changes during the treatment than such as result from the fixation with the vapour of osmic acid on the one hand, and such as may be due, on the other hand, to the slight amount of evaporation of the blood-serum which is inevitable, however rapidly the manipulation is carried out; hence these preparations represent, in my opinion, the nearest approach possible, in the present state of our technique, to the living condition, and may therefore serve as a standard for comparison with the results of other methods. I shall therefore refer briefly in the sequel to these preparations as "the standard," whenever I have to speak of them.

In making the standard preparations I introduced one additional complication into some of them, namely, that on the clean slide a small drop of acidulated methyl-green solution was placed (½ per cent. methyl-green in 1 per cent. acetic acid), and the blood, after exposure to the osmic vapour, was placed on the stain. In the course of a few hours the stain mixes with the blood and tinges the nuclei of the trypanosomes. Comparison of trypanosomes, treated with the acidulated methyl-green (figs. 5–8), with those in which no stain was used (figs. 3, 4), revealed no perceptible difference in size,

[1] I employ for this Czokor's mixture of beeswax and Venetian turpentine, heated together until the mass is hard and smooth (not sticky) when cool, and applied to the edge of the coverslip with a piece of heated wire.

form or general structure,[1] so that no ill-effects result from the use of the stain, while the microscopic image is rendered much sharper and clearer.

My method of carrying out investigations upon the effects of technique was the following: Preparations of Trypanosoma lewisi were fixed and stained in various suitable ways, and the results were all drawn with the camera lucida at a magnification estimated at 3000 diameters, and are reproduced in this memoir at the same scale. It is possible that the figure 3000 is not perfectly accurate, but if not, it makes no difference to the result, since the object was to compare the effects of the methods employed, and for purposes of comparison the exact magnification is immaterial, provided it be uniform throughout. All the drawings given here were executed using always the same microscope and length of tube, the same lenses, camera lucida, drawing board and illumination. Some of them were done by me, but most of them by my assistant, Miss Rhodes, to whom my best thanks are due for her skilful help. Zeiss's apochromatic objective 2 mm., 1·40 aperture, was used, combined with compensating oculars. The source of illumination was the flame of an oil lamp placed edgeways, and concentrated by a Zeiss collecting lens, through a monochromatic screen on to the mirror of the microscope, and thence reflected through a centring achromatic condenser of Zeiss. This illumination, if properly arranged and focussed, gives very perfect definition—a very important point in studying these preparations; I have frequently found that structures had been overlooked which became visible with improved illumination. I frequently compared the results obtained in this way with those given by a monochromatic illuminating apparatus set up with a prism in such a way that any part of the spectrum could be used, in order to obtain confirmation of the structures as drawn with the illumination described.

[1] A slight vacuolation, which is probably an artefact, makes its appearance near the kinetonucleus after treatment with methyl-green (figs. 5–8).

Before proceeding to describe in detail the results of the fixatives and stains used by me, I will say a few words about the methods of applying them. I began with the ordinary method of making smears on slides; this is quite suitable for smears that are to be dried off or fixed with osmic acid vapour, for which the procedure adopted was the following: a glass tube is taken, of suitable size and calibre, and provided with a tightly fitting cork or stopper; at the bottom of the vessel are put twenty drops of 4 per cent. osmic acid solution with one drop of glacial acetic; when the smear has been made, the slide bearing it is placed with as little delay as possible into the tube containing the osmic acid and corked up. It is advisable to take precautions against any part of the blood-smear coming into contact with the osmic acid solution, if one wishes to be economical in the use of this most expensive re-agent. An exposure of thirty to forty-five seconds to the osmic vapour is sufficient; the fixation is practically instantaneous. Slides fixed with osmic vapour in this way may be (1) dried off, then fixed with absolute alcohol or methyl alcohol in the ordinary way; or (2) placed at once without drying into absolute alcohol, stained as desired and then dried off; or (3) fixed, stained, and mounted in Canada-balsam without ever having been dried.

I soon, however, abandoned the use of slides for smears for various reasons. In the first place it is a clumsy method for rapid manipulation, and however anxious one may be to shorten the exposure to the free air, it is very difficult to avoid a certain amount of drying taking place in such large smears, either at the edge or in thin places. In the second place, slide smears do not give good results when the fresh wet smear has to be fixed by sudden immersion in a liquid, which it almost necessarily enters with a dive, so to speak, with one end foremost; in such cases it is common to find all the blood-corpuscles drawn out in an extraordinary manner, and all the trypanosomes stretched out straight in one direction, indicating the line of the dive into the fixative. In the third place I may point out that since the best immersion objectives are

corrected for coverslips, naked smears without coverslips do not give optical results so good as those covered, and that in covered smears it is optically preferable for the smear to be on the coverslip rather than on the slide.

The majority of the preparations described here were done by Schaudinn's method of making smears on a coverslip and then dropping the coverslip flat down plump into the fixative. I hold a coverslip in the fingers of my left hand, a glass rod in my right; in front of me is the liquid to be used for fixation. An assistant draws the drop of blood from a rat's tail, and either places it on the coverslip I am holding, or I take it up on the end of the glass rod from the tail; in either case I smear the blood out with the glass rod and drop the coverslip, with the smear downwards, into the fixative. The whole process is done with one turn of the right wrist and one of the left, far more rapidly than a slide can be manipulated and with much less chance of drying or time for evaporation to take place. Coverslip smears made in this way are always stained and mounted in Canada-balsam without being allowed to dry during any part of the process. They are most conveniently handled in solid watch-glasses (that is to say, a square slab of plate glass, two and a half inches square and a quarter of an inch in thickness, hollowed out on one side into a cavity the size of a watch-glass); the coverslip can be placed in this with only sufficient liquid to keep it wet on the underside (the side of the smear), and can be examined under the microscope with moderate magnification at any time, and the glasses can be stacked one on the top of the other to prevent the contents drying up.

The use of coverslip smears necessitates a modification in the mode of applying the osmic acid vapour for fixation of films. I take a square block of hard paraffin of suitable size, and a glass ring ground flat on the two sides. The glass ring is gently heated and so cemented to the middle of one surface of the block of paraffin, which is then hollowed out into a small cell inside the glass ring. The osmic acid is placed in the cell in the paraffin, and the coverslip, with the blood-smear

downwards, is placed on the glass ring, to which it sticks by means of the wet blood itself, and makes a practically air-tight chamber. A certain amount of the smear has to be sacrificed, of course, by this method, but there is always enough for all practical purposes preserved within the ring. After sufficient exposure the coverslip is lifted carefully off the ring and dropped at once into absolute alcohol or other fixative. In this method the only precaution necessary is the protection of the operator's eyes and air-passages from the vapour of the osmic acid. It is advisable either to wear protective spectacles or to have the osmic cell covered with a glass plate, which an assistant removes the instant it is desired to place a coverslip on it.

Salvin-Moore and Breinl (1907) recommend making smears on slides previously covered with a thin layer of glycerine and albumen, but with what object I do not understand, as the blood-films always stick on perfectly well to the coverslips and slides when plunged suddenly into fixatives, and neither corpuscles nor trypanosomes come off. Even when working with fish-blood, in which the parasites are exceedingly scanty, I have always found them in my never-dried smears just as abundantly as in those done by other methods. In our technique the frail bodies of these unfortunate creatures are subjected to so many processes of violent treatment that to avoid deformation it is better to reduce and eliminate these processes as much as possible rather than to increase them. The addition of glycerine and albumen to the fresh blood containing living trypanosomes can but be an additional source of error, and is therefore, in my opinion, best avoided.

I made several attempts to obtain preparations of trypanosomes by the method recommended by Schaudinn (1902, p. 190) for malarial parasites, that is to say by dropping fresh-drawn blood into fixatives, such as Flemming's fluid or Schaudinn's fluid, in a centrifuge tube, and then carrying out all the subsequent processes of washing, staining, etc., by means of the centrifuge. After much searching I found some

trypanosomes in preparations made in this way, but they were so shrunk and deformed as to be scarcely recognisable.

When preparations that have been mounted in Canada-balsam without being dried at any time are compared with those that have been dried off at some stage of the process, either before fixation, or after fixation with osmic vapour, or after staining, it is found that the trypanosomes in the never-dried preparations are constantly slightly smaller than in the dried-off preparations. Comparison with the standard shows that the dried-off trypanosomes have lost very little in bulk, while the never-dried trypanosomes have lost considerably. I obtained invariably the same results with the trypanosomes and trypanoplasms of fishes; the never-dried specimens mounted in Canada-balsam were always a size smaller than those in the dried-off films. This result proves, in my opinion, that the changes of medium which are incidental to the processes of dehydration with alcohol, clearing with xylol or other media, and mounting in Canada-balsam, have the effect of diminishing the size of the body. So long as it is kept in a semi-fluid plastic condition the body is susceptible to changes of medium, and can be shrunken or swollen by them. If, however, all fluid be once removed from the body by evaporation, it appears to obtain a rigidity of texture which resists further deformation, although it may be altered in form to a greater or less extent by the process of drying itself.

After these general remarks I proceed to describe in detail the effects produced on Trypanosoma lewisi by various fixing and staining methods, using as the standard of comparison the preparations fixed simply with osmic acid vapour and examined in the fluid blood, as described above.

(1) Fixatives.

The fixatives I have used may be grouped conveniently under the following heads: (1) Osmic acid vapour, followed simply by alcohol; (2) mixtures containing osmic acid, (3) mixtures in which corrosive sublimate is the principal in-

gredient; (2) and (3) were either used directly on the wet film, or after previous exposure to osmic acid vapour.

(1) Osmic acid.—I have employed osmic acid as a fixative of the films followed by various other fixatives, such as absolute alcohol, Flemming's fluid, sublimate-acetic, etc. I will only deal in this section with its action when followed by alcohol, and discuss its action when combined with other fixatives in the sections dealing with those fixatives. Of all the methods I have tried, fixation with osmic vapour followed by fixation with alcohol (figs. 60–71) gives the best result as regards size, form, and general characters of the trypanosomes, so far as can be judged from a comparison with the standard (figs. 1–8). The osmic acid was used in the form of vapour given off from a 4 per cent. solution, combined with acetic acid in the following proportions: a 4 per cent. solution osmic acid, 20 drops, glacial acetic acid, 1 drop.

The smears, after exposure to the osmic vapour, were in some cases allowed to dry off, either before further fixation or after staining; but as a rule they were transferred at once to absolute alcohol, and were stained and mounted in Canada-balsam without being allowed to dry at all. I will distinguish these two methods of treatment briefly as the dried-off osmic method and the never-dried osmic method respectively. The dried-off method is most convenient for smears made on slides; for the never-dried methods it is better to make the smears on coverslips. The dried-off osmic smears stain very well by the ordinary Romanowsky methods (Giemsa's stain, azure-erythrosin, etc.), but I have never got good results with the iron-hæmatoxylin stain after drying off. The never-dried smears stain well with Romanowsky stain, and fairly well, but rather faintly, with iron-hæmatoxylin. The details of the flagellum and blepharoplast are difficult to make out after osmic-absolute fixation and iron-hæmatoxylin staining; for these points fixation in sublimate mixtures is far superior.

After the dried-off osmic method the trypanosomes do not show what I will term briefly a "periplast-line," that is to say, a clear border under the periplast, causing the periplast

to stand out distinctly from the body (see below). By the never-dried method, however, a periplast-line appears very distinctly in the iron-hæmatoxylin preparations (Pl. 21, figs. 10, 11). Its appearance in the preparations stained with the Romanowsky stain depends largely on the degree to which the stain has been extracted by the acetone during the process of mounting in Canada-balsam, and the result varies greatly in one and the same slide (Pl. 22, figs. 64–69); a matter which I shall discuss more fully in the section dealing with this stain and its effects. One of the most constant results of the process of drying off is seen in the relations of flagellum, blepharoplast and kinetonucleus. In never-dried preparations the flagellum starts from close to the kinetonucleus, with which the blepharoplast is nearly in contact. In dried-off preparations, on the other hand, there is a distinct interval between blepharoplast and kinetonucleus, almost equal to the width of the latter in many cases (Pl. 22, figs. 60, 61). The same result is seen in preparations in which osmic fixation is followed by Flemming's fluid or other reagents, and dried off after staining with Romanowsky's stain, and also in preparations dried before fixation. It follows, therefore, that the effect of drying is to draw apart kinetonucleus and blepharoplast, probably as the result of a longitudinal contraction in the flagellum or in its basal portion.

(2) Osmic acid mixtures.—The two famous combinations, Flemming's fluid and Hermann's fluid, were used, the former both direct on the wet film and after previous exposure to osmic vapour, the latter only directly on the film. The Flemming's fluid used was the so-called strong mixture; the Hermann's fluid was made up in the same manner, and differed only in the substitution of platinum chloride (so-called), 1 per cent., for the chromic acid, 1 per cent. A variety of staining methods was tried on these films; I will describe the results obtained by the various stains in the sections dealing specially with them below. The best results were obtained with iron-hæmatoxylin, and the following account of the action of these two fixatives refers more particularly to

preparations stained by this method, unless the contrary is stated.

When used directly on the wet films both fixatives gave very similar results. The trypanosomes are under-sized and deformed, appearing too short in proportion to their breadth, as if contracted in the longitudinal direction (figs. 36–38, 40–44); their attitudes in the preparations look strained and unnatural, as if their end had not been a peaceful one. The amount of the deformation is distinctly greater after Hermann's fluid than after Flemming's, and with the latter re-agent it varies in different preparations. I am inclined to think that the amount of deformation is related in an inverse manner to the degree to which the films have lost moisture previous to fixation. However carefully and rapidly the preparation of the film be carried out, it is clear that a thin film of blood must lose a certain amount of moisture before it reaches the fixative. My impression is that the more the film dries before coming into the fixative, the less the trypanosomes undergo shrinkage and distortion of the body-form.

As regards minuter details, the following points are to be noted: The body of the trypanosome does not show a periplast-line; the nucleus has an appearance most characteristic of the effects of these fixatives; it is surrounded by a clear space, often very distinct, but not sharply limited (figs. 36–38, 43, 44), which I regard as the result of shrinkage. In the nucleus the karyosome is very distinct, and frequently also other coarse chromatic granules are to be seen. A similar condition of the nucleus could be made out also in trypanosomes stained with all the other staining methods that were used, and must, therefore, be attributed to the action of the fixative.

The kinetonucleus and blepharoplast appear normal after Flemming's and Hermann's fluids, but the flagellum often appears extremely crinkled, with sharp bends and angles, very different from the normal smooth undulating curves; its appearance is strongly suggestive of longitudinal shrinkage.

In extreme cases (Pl. 21, fig. 38) the appearance of the flagellum recalls that of a wire that has been untwisted from the neck of a soda-water bottle.

In films fixed with osmic acid vapour before being put into Flemming's fluid, I find but little difference from those fixed with absolute alcohol after the osmic treatment, except that the trypanosomes are rather broader in proportion to their length (figs. 80, 81). The nucleus stained with Giemsa's stain is a large red patch, as large as, or even larger than, the whole clear space seen after direct fixation with Flemming's and Hermann's fluids. When stained with iron-hæmatoxylin, after Flemming's fluid preceded by exposure to osmic acid vapour (fig. 39), the nucleus of the trypanosome does not show the clear space round it described above, but appears as a broad oval or nearly circular in outline. The body shows no periplast-line and the flagellum has the normal appearance. Previous exposure to osmic acid vapour would appear, therefore, to obviate some of the defective results obtained by the direct action of Flemming's fluid.

(3) Sublimate mixtures.—Three different sublimate mixtures were used for fixation: (1) Sublimate and acetic acid, (2) Schaudinn's fluid, (3) Mann's picro-corrosive mixture. All three were used directly on the wet film. The first two were used also after previous exposure of the wet film to osmic vapour. In all cases the fixing solution was allowed to act for about half an hour on the film.

(a) Sublimate acetic.—This was used in the proportion of 95 parts (by volume) of corrosive sublimate solution, saturated in distilled water, and 5 parts of glacial acetic acid. A mixture was also tried of 99 parts corrosive sublimate solution to 1 part of glacial acetic, but was found to produce very marked shrinkage in the trypanosomes (Pl. 21, figs. 12, 13) and was not used again.

The fixative was used either directly on the films (figs. 19–22, 82–84, 88–91), or after previous exposure to osmic vapour (figs. 14–18, 75), in both cases without drying before putting in the sublimate solution. The fixed films were stained in

various ways, principally with the Romanowsky stain, with iron-hæmatoxylin, and with Twort's stain. Some of the films stained by the Romanowsky method were dried off (Pl. 22, figs. 70, 73), but as a rule they were kept wet and passed through acetone and xylol into balsam. All films stained with iron-hæmatoxylin or with Twort's stain were kept wet throughout and mounted in balsam.

After previous fixation with osmic vapour the trypanosomes generally show a distinct periplast line (figs. 17, 18, 75), not to be seen when the sublimate mixture is used directly. There is little other difference to be noticed between osmic-fixed films and those in which osmic has not been used. With either method iron-hæmatoxylin gives good results; Twort's stain, however, does not work well after osmic fixation, but stains very well after fixation direct in the sublimate mixture (figs. 82–84).

(b) Schaudinn's fluid (two volumes of saturated solution of corrosive sublimate in water, one volume of absolute alcohol, with the addition of a few drops of glacial acetic).—This fluid was also used with (figs. 23, 24) or without (figs. 25–28, 76, 85) previous exposure of the film to osmic vapour. The films were never dried and were mounted after staining in Canada-balsam. The results were on the whole similar to those observed with sublimate acetic. A periplast-line can generally be observed, and appears to depend on the degree of extraction of the stain, as is well seen after iron-hæmatoxylin (figs. 23–38). Twort's stain gives very good results if the fixing fluid be used without previous osmic vapour-fixation (fig. 85).

(c) Mann's picro-corrosive.—Made up as follows: 2·5 grm. of corrosive sublimate dissolved in 100 c.c. of boiling distilled water; when dissolved, 1 grm. of picric acid added; the mixture allowed to cool, and either used as made up, or with addition of 15–20 c.c. of formol, mixed in immediately before use. Wet films were fixed in the mixture and stained in various ways without drying at any time. I could perceive very little difference resulting from the presence or

absence of formol, but got the impression that the fixation was rather better, and the stain sharper, after use of the mixture containing formol.

Mann's fluid gave me very uniform results. The trypanosomes show no periplast-line and appear slender. The blepharoplast and flagellum show up with remarkable clearness, especially after iron-hæmatoxylin, and this appears to me the method of choice for the study of these structures.

With all the sublimate-containing mixtures that have been tried, a marked diminution of size is apparent in the never-dried preparations as compared with the type (compare especially figs. 1–8 with figs. 16, 35, and 79). The shrinkage is on the whole greatest after the picro-corrosive mixture; the disappearance of the periplast-line after fixation with picro-corrosive indicates, perhaps, a certain amount of collapse in the cytoplasmic body.

As already pointed out above, the shrinkage is scarcely perceptible if the trypanosomes have been allowed to dry off after exposure to the osmic vapour before fixation in the sublimate mixture. Figs. 70 and 73 show this plainly when compared with figs. 14, 15, 16, etc.

The facts indicate further that the shrinkage is due not only to the action of the fixative but also to the subsequent processes which the trypanosomes pass through, namely, first the staining process, secondly the dehydration and clearing preparatory to mounting in Canada-balsam. It can be observed, for example, that the Romanowsky stain, when used on trypanosomes which have not been dried, seems to shrink them more than do other stains, such as iron-hæmatoxylin or Twort's stain; compare, for instance, figs. 19, 20, 82–84 with figs. 21, 22, from a film which, after having been fixed and stained in Giemsa's stain without drying, was dried off after the stain; the trypanosomes are shrunk to a ludicrous extent in the latter case.

Although the trypanosomes are distinctly diminished in size and general proportions after sublimate fixation (when never dried), the shrinkage appears to take place evenly, and

the trypanosomes are scarcely if at all deformed. The body maintains an even, slender form, differing only from the type in size. The minute details of the nuclei, blepharoplast, and flagellum are very well seen after sublimate mixtures, and especially after picro-corrosive, better, indeed, than with any other fixative in my opinion.

(2) Staining Methods.

I have made trial of a number of staining methods on films preserved in various ways, and have obtained results which may be considered from two points of view. Some staining methods give good results generally, and yield what I may term "show" preparations — that is to say, preparations which one would not be ashamed to demonstrate to students or to strangers. Other stains give results which are quite useless considered as preparations for purposes of demonstration, but which may be quite interesting as micro-chemical reactions. I will discuss in detail the three staining methods that have given me the best results generally, namely the Romanowsky stain in its various modifications, Heidenhain's iron-hæmatoxylin method, and Twort's combination of neutral red and licht-grün; after which I will deal briefly with other stains I have tried.

(1) Romanowsky stain.[1]—In applying this method I have always used Giemsa's modification; I obtain the stain made up in the fluid condition from Grübler, and for actual use I mix the stain with distilled water in the proportion of one drop of the stain to 1 c.c. of water. The preparations are left in from three to eighteen hours. Osmic-fixed preparations are apt to stain very darkly, and require a much shorter time than those simply fixed with alcohol. The preparations when taken out of the stain are washed with distilled water, treated for a few seconds with Unna's tannin-orange (obtained from

[1] I use the term "Romanowsky stain" as a general term for the combination of stains, of which the methods of Leishman, Giemsa, etc., are special modifications, in application or substance.

Grübler), and then washed in a current of tap-water for a minute or two. After this the preparation is washed with distilled water, and either dried off, or passed rapidly through three changes of acetone into xylol, and mounted in Canada-balsam.

Some preparations were also stained for me by Dr. J. D. Thomson with a mixture of azure and erythrosin in the following manner: A mixture was made of (1) azure I, 1 per cent., in equal parts of glycerine and methyl alcohol, 1 volume; (2) erythrosin, 0·1 per cent., in 0·25 per cent. formol, 2 volumes; (3) distilled water, 8 volumes. The two staining fluids, kept ready in solution, are mixed with the water immediately before use, and the mixture is allowed to act for from half an hour to one hour; it works better if kept at about blood-temperature. After staining, the preparations are either washed with distilled water only, or also with tannin-orange followed by tap-water, as described above, and then either dried off or mounted without drying in Canada-balsam, passing through acetone and xylol.

The azure-erythrosin mixture stains the trypanosomes in colours slightly different from that resulting from Giemsa's stain. The body is more purple in tint, and the red colour of the nucleus and flagellum appears rather deeper after azure-erythrosin. The general effects of the two methods are the same, and do not require to be discussed separately.

The Romanowsky stain is undoubtedly the best method for studying the general characters of the trypanosomes, especially if combined with osmic fixation, which, as I have shown, preserves very well the form and structure of the body and results in the minimum of shrinkage and deformation. I have always got the best results after osmic vapour followed by absolute alcohol; when the osmic is followed by other histological reagents, such as Flemming's fluid or sublimate-acetic, the results are inferior, but still usually quite good. On the other hand I have never got good preparations with the Romanowsky stain after wet fixation in hardening fluids without previous exposure to osmic vapour; the trypanosomes

become shrunk and deformed, often very greatly, and the stain is blotchy and irregular. That has been my experience in all cases with smears fixed in Flemming's fluid, sublimate-acetic, Schaudinn's fluid, Mann's fluid, etc., stained with Giemsa's stain, and mounted without drying in Canada-balsam or dried off. Preparations made in this manner are usually quite useless, while the companion slides, stained with iron-hæmatoxylin and mounted in the ordinary way, are very good.

The Romanowsky stain has the further advantage of being easy to apply and comparatively rapid in action. It is therefore the method of choice for workers to whom time, or laboratory equipment, are material considerations—that is to say, to those dealing in a limited time with large quantities of material, or working under disadvantages of installation, as in the tropics. Nevertheless I shall try to show that, as Schaudinn[1] has already stated clearly, the Romanowsky stain has some glaring defects in its action on minute structural details, and that some false conclusions and interpretations have been based upon its results. It is very important that its effects should be studied carefully and compared with those yielded by other methods, for only in this manner is it possible to eliminate errors and to discount false appearances produced by it, and to obtain, so to speak, a constant formula for estimating and interpreting the results of its use.

The peculiarities of the Romanowsky method of staining, as regards its effects on minute structures, are at once apparent when trypanosomes stained by this method are compared with others, fixed in precisely the same manner, but stained with iron-hæmatoxylin. The differences are very great, and often very puzzling; compare, for example, fig. 73, and figs. 14, 15; figs. 80, 81, and fig. 39; figs. 62, 63, and figs. 10, 11. To begin with the nuclear structures, the kinetonucleus appears

[1] "Nur ist sie (die Romanowsky'sche Färbung) mit Vorsicht zu benutzen, weil sie oft überfärbt und Strukturen vortäuscht, die garnicht vorhanden sind. Ohne Kontrolle durch Hämatoxylin darf man keine Schlüsse über Kernstrukturen bloss nach Romanowsky-Präparaten ziehen." Schaudinn (1902), p. 191.

much larger after the Romanowsky stain than it does after iron-hæmatoxylin, perhaps four times as large; the same two types of form are to be made out in the kinetonucleus, but they have become magnified, as it were, in the former case. The trophonucleus is not only much larger after staining by the Romanowsky method, but its structure appears quite different from that seen after iron-hæmatoxylin and other methods. In the place of a simple oval vesicle-like structure containing a deeply staining karyosome, we find after the Romanowsky stain an opaque mass of deeply stained coarse grains of reddish colour, in which a karyosome often cannot be made out with certainty. Sometimes the stained grains are closely packed and scarcely distinguishable individually; sometimes, and especially in preparations dried before fixation, the grains appear separate and distinct. Very frequently the nucleus shows a clearer spot at or near the centre (figs. 70, 80, 81). The flagellum appears thick and coarse and the blepharoplast is usually fairly large and distinct after the Romanowsky stain.

What is the explanation of these differences? The large size of the kinetonucleus and trophonucleus and the thickness of the flagellum after the Romanowsky stain are shown by the standard (figs. 1–8) to be a case of actual enlargement or thickening, not to be explained by supposing that after other methods, such as the iron-hæmatoxylin stain, these structures are shrunk. Such an enlargement can only be explained by a tendency of the red stain or stains, which are active in the Romanowsky combination, to deposit not only in, but also around, the structures that are coloured by them. Comparison of the kinetonucleus, in preparations stained by the Romanowsky method and by other methods, is alone sufficient to prove conclusively that the enlargement is due to this cause, and gives a clue to the interpretation of the very remarkable and perplexing differences seen in the structure and appearance of the trophonuclei in the two cases.

In order to discuss the effects of the Romanowsky stain on the trophonucleus, it is necessary that I should anticipate here

the conclusions to which I come further on in this memoir with regard to the minute structure of this body in Trypanosoma lewisi. The trophonucleus is a rounded oval body with a distinct limiting envelope, which is not to be regarded as a true nuclear membrane in the sense in which this term is used for metazoan nuclei, but, in all probability, as a condensation at the periphery of the chromatin-substance itself. Inside this envelope is a space, filled doubtless in the living animal by a nuclear sap, in which are contained other chromatin-bodies; first of all the conspicuous karyosome, sometimes double or further subdivided, which in its staining reactions resembles the kinetonucleus, and appears of dense texture; secondly, smaller granules of chromatin, some of which may be fairly large and plainly visible, but which for the most part appear to be minute and not capable of being resolved by the microscope as distinct structures, but are scattered in a state of fine division throughout the nuclear sap, giving to the trophonucleus the even dark grey tint which it shows in an iron-hæmatoxylin preparation at a certain degree of extraction, or the pale red tint which it exhibits after Twort's stain.

Applying the conclusion stated above, namely, that the red stain of the Romanowsky mixture deposits round the structures it stains, to the conception of the structure of the trophonucleus that I have put forward, we can explain the results of the stain as follows. The deposition of the stain round the nuclear membrane accounts for the apparent increase of size of the nucleus as a whole. At the same time the stain is deposited round the minute granules of chromatin within the nucleus, enlarging them to relatively coarse grains. The karyosome doubtless shares also in the enlargement, but is generally obscured by the closely packed and secondarily enlarged chromatin grains, and is consequently very difficult to make out clearly.

In support of the above conclusion I refer to the series of figures drawn from three preparations (*a*, *b*, *c*) which were made at the same time from the same rat, and which were all

fixed in the same manner, namely by osmic vapour followed by absolute alcohol, without drying at any time ; *a* (figs. 64–69) and *b* (figs. 62, 63) were stained with Giemsa's stain, and passed through acetone and xylol into Canada-balsam ; *c* (figs. 10, 11) was stained with iron-hæmatoxylin and mounted in the ordinary way.

In preparation *b* (figs. 62, 63) the passage through the acetone was evidently effected without any extraction of the stain taking place ; the trypanosomes are perfectly similar in general appearance to those stained with Giemsa and dried off (figs. 60, 61). In preparation *a* (figs. 64–69), on the other hand, a considerable amount of extraction has taken place, which, however, is different in degree in different parts of the preparation ; in one place a clump of trypanosomes may be found, all showing a deeply stained, opaque red trophonucleus ; another patch of trypanosomes will show the stain greatly extracted. Hence in this preparation I have been able to find every stage of the extraction of the stain, and have put together the series shown in my figures.

The series begins with trypanosomes, in which the trophonucleus differs only from those in preparation *b* by its smaller size (fig. 64), showing that the process of extraction begins by removing the stain deposited outside the nuclear membrane ; at the same time the kinetonucleus is considerably diminished in size, the periplast-line begins to stand out, and the flagellum is thinner. In the next stage (fig. 65), the trophonucleus is pale with a few scattered coarse granulations, amongst which it is still difficult to identify the karyosome. In the next two figures (figs. 66, 67), it is seen that the coarse granulations are disappearing, leaving the karyosome standing out plainly, with a more or less distinct clearer space round it. In the last two figures (figs. 68, 69), which represent the last stage of extraction and the condition that is most frequently met with in the preparation, the trophonucleus is reduced to an oval space of faint pink colour, in which the karyosome or karyosomes stand out sharp and clear—exactly the same state of things which is found in preparation *c*

(figs. 10, 11), stained with iron-hæmatoxylin, and which results also from other stains, such as methyl-green, Twort's stain, etc. The resemblance between preparation *a*, when fully extracted, and preparation *c*, extends also to other points. The flagellum is fine and delicate in structure, the blepharoplast minute and scarcely visible, and the periplast-line stands out sharply on the concave sides of the body. But there is one remarkable point which puzzled me greatly, and which I took great pains to assure myself was real and not due to errors in representation, namely, that in preparation *a* (figs. 64–69), the trypanosomes are constantly smaller as a whole than in preparation *b* (figs. 62, 63). This is difficult to explain. It may be that the preparation *b* has dried slightly during the process of fixation, and so resisted the tendency to shrinkage, which I believe must always be reckoned with in preparations mounted without drying in Canada-balsam. But this is evidently not the whole of the explanation, for the fully extracted trypanosomes in preparation *a* (figs. 68, 69) are smaller than those in *c* (figs. 10, 11), stained with iron-hæmatoxylin. It seems to me quite possible that when a considerable quantity of stain deposited in the body of the trypanosome is dissolved out, the body may shrink to some extent as the substance of the stain is removed. I do not know how else to explain the distinctly smaller size of the trypanosomes in question.

There is one further point relating to the nucleus which I find very difficult to explain, namely, the frequent appearance, in trypanosomes stained with Giemsa's stain in the ordinary way, of a lighter spot at or near the centre (figs. 61, 70, 80, 81, etc.). From a comparison with preparations in which the stain is more or less extracted, this clear spot appears to correspond to a space or chromatin-free area close beside the slightly excentric karyosome; it does not appear, however, to be always present.

I pass to the consideration of the action of the Romanowsky stain on the flagellum and periplast. It is well known that both these structures stain red with the stain. Schaudinn

first, and after him Prowazek and others, have attributed this to a similarity in origin and substance between the locomotor apparatus and the nucleus. According to Schaudinn (1904, p. 395) the entire locomotor apparatus (Trypanosoma noctuæ) is a nuclear product, and the periplast stains red like the nucleus, because it contains myonemes originating from the mantle-fibres of a nuclear spindle. Since Schaudinn wrote, the staining properties of the flagellum have generally been considered as due to its affinities with the chromatin of the nucleus. This is a good instance of the danger of founding conclusions of this sort on the results obtained by a single staining method; for when trypanosomes are stained with Twort's combination of neutral red and Licht-grün, the flagellum, blepharoplast and periplast stain green, in sharpest contrast to the two nuclei, which are both stained red (Pl. 23); hence this method leads to conclusions diametrically opposite to those that can be drawn from the results given by the Romanowsky combination.

I cannot claim to have an expert knowledge of the chemical changes that go on, and the staining substances that result from them in the Romanowsky mixture; but certain conclusions seem to me legitimate, and, indeed, quite obvious. In the first place the mixture contains eosin, or its equivalent, erythrosin. Eosin is not, however, a stain for chromatin, and it cannot be held accountable for the red coloration of the chromatin. When trypanosomes are stained with methylene blue and eosin by Chenzinsky's method, the chromatin stains a pure blue colour. It is generally acknowledged that there is, in the Romanowsky combination, a red stain other than eosin at work which is accountable for the red stain of the chromatin. If it be true, however, that in the Romanowsky mixture at least two red stains are present, the one a stain for chromatin, the other not, it is evident that the fact of two structures being stained red by the mixture is no proof whatever of any similarity in nature or substance between them. Hence the red stain of the flagellum and periplast is not proof in itself of their relationship to chromatin.

(2) Heidenhain's iron-hæmatoxylin.—For this stain I make use of 3½ per cent. solution of iron-alum in distilled water and ½ per cent. solution of hæmatoxylin, which is made up as follows: A stock solution is made in the proportions of 1 gramme hæmatoxylin, 10 c.c. absolute alcohol, 90 c.c. distilled water; for use the stock solution is mixed with an equal volume of distilled water.

In agreement with Schaudinn (1902, p. 190), I found it necessary to let the mordant and stain act for a long time. My procedure is as follows: Films, however fixed, are brought into absolute alcohol and kept there for an hour or so, then brought down through a series of grades of alcohol, differing by 10 per cent. between two consecutive grades, into distilled water, thence into the iron-alum solution, in which they are left till the next morning. The films are then dipped for an instant into distilled water, transferred to the hæmatoxylin solution, and left for at least twenty-four hours. I use both the iron-alum and hæmatoxylin solution in the solid watch-glasses mentioned above, taking care that none of the solution, in either case, gets on to the clean upper surface of the coverslip.

The important part of the whole method is the process of extraction of the stain. After being in the hæmatoxylin the film is washed with distilled water and placed in the iron-alum solution; in a short time clouds of colour can be seen coming out, especially if the coverslip be gently moved; it is then taken from the iron-alum and placed in tap-water, which stops the process of extraction. I now examine the film, in tap-water in a watch-glass, with a dry lens (Zeiss D with oc. 4). If the karyosomes of the trypanosomes can be seen clearly the extraction is sufficient, if not the film is put back again into the iron-alum for a short time, and the process of extraction and examination repeated. When the extraction is judged to be sufficient, the films are washed for twenty minutes in a current of tap-water, which is easily done by letting them float on the surface of the water in a beaker through which a gentle current of water is passed from a

pipe immersed below the surface of the water; the cover-slip will neither sink nor run over the edge of the beaker with the current, and can be washed with safety for any length of time. After the tap-water I put them for a few minutes in distilled water, then pass them up through the grades of alcohol into absolute, in which I leave them for at least an hour, then pass them through xylol into Canada-balsam.

As a rule I have always done several films at the same time, and extract the stain to different degrees in different films (compare figs. 25–28, 29–32). When the trypanosomes come out of the hæmatoxylin they appear uniformly black all through. The stain first comes out of the general cytoplasm; next out of the cytoplasmic granules and the body of the nucleus, leaving the karyosome sharp; with more extraction it is taken out of the flagellum and blepharoplast; the bodies that retain it the longest are the kinetonucleus and karyosome, especially the former. In fish-trypanosomes I was able, in some instances, to see myonemes after moderate extraction when the stain was only removed from the general cytoplasm; but in Trypanosoma lewisi I have never succeeded in seeing myonemes.

Iron-hæmatoxylin is the stain of choice for minute structural details of the nuclei and locomotor apparatus. Perfect reliance can, in my opinion, be placed on the results yielded by it, and the stain can always be trusted to give uniform results with the same degree of extraction. It stains most sharply and clearly after sublimate mixtures, less so after osmic vapour and alcohol simply. I have never had good results with it in films dried at any time, before or after fixation. I have also never found any advantage, so far as trypanosomes are concerned, in counter-staining with eosin, orange, etc.; all such processes appear to me simply a waste of time.

(3) Twort's stain.—This is a combination of neutral red and Licht-grün invented by Dr. Twort, of the Bacteriological Laboratory, London Hospital Medical College, who has

kindly furnished me with the following directions for making up and using the stain:

"To prepare the stain make up half-saturated watery solutions of neutral red and of Grübler's light green, using distilled water. Place the neutral red solution in a large open vessel, and add to it sufficient light green solution to combine with the neutral red; the compound will form a precipitate. It is better not to have an excess of either stain, as the precipitate is difficult to wash. When the neutralisation-point is reached, the water will contain a small quantity of both dyes in solution, giving it a dark appearance; the presence of both stains in solution can be easily tested by dropping a drop of the filtrate on to blotting paper; this will spread out, leaving a light red central zone and a faint green outer zone.

"To collect the precipitate it is better not to filter, for the stain soon blocks the pores of the filter-paper. If the solutions are warmed to about 30° or 40° C. before mixing, the precipitate will form in large sticky masses, some of which containing air-bubbles float to the surface and can be removed with a spatula. The rest settle and stick to the bottom and sides of the vessel, and can be collected after pouring off the fluid. The precipitate so collected is rinsed in distilled water and dried at 37° C. In this state it forms dark greenish masses, insoluble in water, somewhat soluble in ethyl alcohol, but soluble to a greater extent in methyl alcohol, and especially so if 5 per cent. of glycerine is added.

"To make up the stain for use, it is best to pound up about 0·25 grm. of the stain with some clean, sharp sand; this prevents the stain going into a sticky mass when the alcohol is added. To the powder so obtained is now added some purest methyl alcohol, acetone-free, containing 5 per cent. by volume of glycerine. Pound up well to obtain a saturated solution; then pour off and add a further quantity of alcohol glycerine solution and repeat the pounding; about 100 c.c. stain can be made from the quantity given.

"The alcohol-glycerine mixture dissolves about ·1 per cent.

of the stain, but it is always better to work with an excess of the powder when grinding up, otherwise it is very difficult to obtain a saturated solution.

"The solution when filtered should be kept in a good-stoppered bottle (and if a completely saturated solution has been obtained add 10 per cent. more alcohol-glycerine mixture).

"Stain for five or ten minutes with the stain made up by mixing one part of distilled water with two parts of the glycerine-alcohol stain-solution. Rinse in distilled water. Fix for half to one minute in Unna's glycerine-ether mixture, 2 per cent. in distilled water. Rinse in distilled water.

"Differentiate and dehydrate in absolute alcohol. Should there be much precipitate this can easily be removed by a few drops of methyl alcohol or equal parts of absolute alcohol and xylol. Remove absolute alcohol with xylol and mount in Canada-balsam in the usual way."

I made a number of smears of blood containing T. lewisi in order to try the effects of Twort's stain, and also used the stain mixed in different proportions with distilled water, and allowed to act for varying lengths of time. I could not obtain good results with any smears fixed with osmic acid, either when smears previously exposed to osmic vapour were subsequently treated with absolute alcohol or sublimate-acetic or when smears were fixed direct in Flemming's or Hermann's fluids. On the other hand I got excellent results with smears fixed direct in sublimate mixtures, either sublimate-acetic, Schaudinn's fluid, or Mann's picro-corrosive, with or without formol (Pl. 23). As regards the application of the stain I did not obtain good results with weak mixtures used for a long time, but I got my best results by mixing the stain in equal quantity with distilled water, or by using two or even three parts of stain to one of distilled water and allowing the stain to act for from twenty minutes to an hour. I differentiated with 5 per cent. Unna's glycerine-ether solution. I found that when the staining mixture was placed on the slide or coverslip a very dense precipitate formed on the smear, but by placing the stain in a suitable vessel such as a watch-glass,

and putting the slide or coverslip in it with the smear downwards, no precipitate was formed on the smear.

My recommendations for the use of this stain for trypanosomes are, therefore: fix with sublimate mixtures; use the stain strong, 50–75 per cent.; stain for an hour, more or less; and place the smear downwards in the stain.

Twort's stain, for "show" preparations, has the fault that it stains rather faintly; this might, perhaps, be overcome by letting it act longer. On the other hand it gives results which are extremely instructive and important (Pl. 23). The trypanosomes are stained in two colours, red and green. The two nuclei and the chromatoid granules are red; the flagellum, blepharoplast, and periplast are green. The general protoplasm appears to have a greenish tint, perhaps due to the periplast. The structure of the trophonucleus and the size of the kinetonucleus are just as they are after iron-hæmatoxylin.

Other stains.—I made trial of a number of other stains, or combinations of stains, none of which gave results which could permit me to recommend their use for "show" preparations, but in some cases the results are interesting as reactions.

Delafield's hæmatoxylin.—Stock-solution made up as recommended in Bolles Lee's 'Vade-Mecum' (5th edition, p. 184). For staining, a few drops added to about 30 c.c. distilled water, acidulated with one drop of glacial acetic acid; films stained in it for several hours, then washed with distilled water, brought up through the grades of alcohol into 90 per cent.; left in this overnight, then mounted in the usual way.

Effects (after Hermann's fluid) very poor; the trophonucleus a narrow dark patch with a clear space round (effect of fixative, see above); kinetonucleus indistinct; flagellum faintly stained; cystoplasmic granules rather deeply stained, giving the body a blotchy, marbled appearance.

I think it highly probable that Delafield's hæmatoxylin would have given better results after other fixatives. I have recently obtained excellent results with it, on other material,

after fixation with Schaudinn's fluid and Mann's fluid; but as the preparations stained with it are so inferior to those obtained with iron-hæmatoxylin, it did not seem to me worth while to waste time on experimenting with it.

Gentian violet and safranin.—I used these two stains made up as follows: 1 grm. of the stain in 90 c.c. aniline water with 10 c.c. absolute alcohol (Hermann's formula).

Films fixed in Hermann's fluid or Schaudinn's fluid, and stained in gentian violet for half an hour or so, were washed with absolute alcohol, then extracted in absolute alcohol saturated with orange G, then mounted in Canada-balsam after passage through xylol.

Result (fig. 40): Trophonucleus a dark patch with karyosome not distinct; kinetonucleus indistinct; flagellum faintly stained; cytoplasm showing the very same appearance as after Delafield's hæmatoxylin.

Other films, fixed as before, were stained in safranin at least twenty-four hours, washed with absolute alcohol, acidulated alcohol, and absolute alcohol again in rapid succession, then stained in gentian violet and differentiated with orange as already described.

The only effect (figs. 41, 42) produced by the additional treatment with the safranin was to deepen the staining of the cytoplasmic granules, which assume a brownish, muddy tint, and so increase the marbled, blotchy appearance of the cytoplasm.

Methylene blue and eosin.—Used according to Chenzinsky's method, as given in Bolles Lee's 'Vade-mecum' (5th edition, p. 222); films fixed with Hermann's and Schaudinn's fluids. Grübler's methylene blue BX was used.

Result: Exceedingly poor as show preparations, but interesting as showing the two nuclei and the cytoplasmic granules stained blue; no other parts of the body are stained at all, hence the trypanosomes appear shadowy and ghost-like, recognisable at first only by their nuclei and granules, but after careful examination the other parts of the body can be made out. The trophonucleus appears as a blue patch, with

the karyosome not very distinct; the kinetonucleus is sharp and deeply stained, its size the same as after iron-hæmatoxylin. The body shows blotchy blue granules, varying in amount in different specimens.

Carmine stains.—I have tried picrocarmine and other carmine stains on trypanosomes, but have never obtained any results worth mentioning.

One cause of the defective results given by some of the stains mentioned above—for instance, Delafield's hæmatoxylin, safranin, gentian violet—seems to me to be in the fact the stain colours the cytoplasmic granules so deeply as to obscure other parts, especially the trophonucleus, which stains less deeply.

It must, I think, be the experience of every one who has tried different methods of technique on different objects, as it certainly has been my experience, how impossible it is to infer or predict the results that a given method will yield for a particular object from the results that it yields on other objects. This truth is constantly brought home to anyone studying trypanosomes, as one so frequently obtains preparations in which the trypanosomes are vile, while the leucocytes are beautifully stained. Thus in films stained with Twort's stain after Hermann's fluid, the leucocytes leave nothing to be desired, while the trypanosomes are useless. Similarly in my preparations stained with methylene-blue-eosin the leucocytes are exquisite.

I have had some experience of the results of technique on another class of objects which might be expected to be not so different from trypanosomes in their reactions, namely, the collar-cells of sponges. A collar-cell is a flagellate organism which might be described as possessing a non-undulating membrane, perhaps similar, morphologically, to that of trypanosomes, but not connected with the flagellum and not contractile in the same manner, only slowly retractile. No *à priori* reason presents itself to me that would lead me to expect a trypanosome and a collar-cell to be very different in their staining reactions.

Beautiful preparations of collar-cells can be obtained by fixation in osmic acid followed at once by staining with picrocarmine; the same method is absolutely useless for trypanosomes. The osmic-picrocarmine method shows the nucleus of the collar-cells as a deeply stained pink mass, in which a nucleolus can just be made out, but beautiful preparations of nuclear structure in collar-cells can be obtained by fixation in Flemming's or Hermann's fluids followed by Delafield's hæmatoxylin, safranin, gentian violet, methylene-blue-eosin, etc.—just the methods, in fact, which I used on trypanosomes with such ill success (but with excellent results on the leucocytes).

It seems to me, therefore, quite illogical to argue that a method is good or bad for trypanosomes or any other cells, because it is good or bad for some quite distinct class of object. Every kind of cell requires its own special technique, which must be established empirically by trial, and can be discovered only to very limited extent and with great uncertainty by analogy.

Part II.—The Structure of Trypanosoma Lewisi.

(1) General Structure, Form and Dimensions.

As I have stated above, I believe that the preparations fixed simply with osmic acid vapour, and examined in the blood with or without addition of acidulated methyl-green but without further treatment of any kind, represent the nearest possible approach to the living condition in form and dimensions. In such "standard" preparations (figs. 1–8) the body of the trypanosome appears long and slender, sharply marked off from the distinct undulating membrane and flagellum. The kinetonucleus is seen near one extremity, which for convenience may be termed "posterior." The trophonucleus is seen as an oval, well-defined space, containing the distinct karyosome, and situated at rather more than two thirds the length of the body from the posterior end. We

may, therefore, for purposes of description, divide the body into three regions: the pre-nuclear region in front of the trophonucleus; the inter-nuclear region between the two nuclei; and the post-nuclear region behind the kinetonucleus.

In the inter-nuclear region the body is roughly cylindrical, but slightly thicker midway between the nuclei. In the pre-nuclear region the body tapers very rapidly to a fine filamentous prolongation, often difficult to distinguish from the flagellum. In the post-nuclear region the body also tapers evenly and rapidly to a point.

The flagellum arises from the blepharoplast or basal granule situated in front of the kinetonucleus, and runs at once slantingly forwards up to the surface of the body to form the marginal flagellum, running along the edge of the undulating membrane and continued beyond the anterior extremity as the free flagellum. The breadth of the undulating membrane appears greater or less, according to the view presented by the trypanosome; so far as can be judged, it is rather more than half the greatest thickness of the body. In this trypanosome the undulating membrane is not much pleated and runs in even, sinuous curves, corresponding to the twists of the body. In any preparation the trypanosomes are found in all sorts of positions, but, speaking generally, the body usually shows one or two main curves, and may be briefly described as either more or less C-shaped or S-shaped. In either case the undulating membrane keeps always to the convexity of the curves, crossing over the body between each bend. The explanation of this is to be found in the fact that the marginal flagellum is considerably longer than that portion of the body along which its runs, as can be determined easily by actual measurement; it is shown in the table of measurements given below that the average length of the pre-nuclear and inter-nuclear regions together is 19·26 μ, while the average length of the marginal flagellum is 23·51 μ. If we imagine the marginal flagellum as an elastic fibril joined by the undulating membrane to a considerably

shorter body, plastic and (after death) inert, we can understand the body being thrown into curves by the elasticity of the flagellum, which, being longer, necessarily runs on the outside of the curves. Only in a single instance have I seen the marginal flagellum on the concave side of a curve in a preparation of T. lewisi (fig. 9), and in this case the flagellum showed a double bend with sharp angles at this point, suggesting a forcible pleating of the flagellum, owing to the fact that the body, wedged in between blood-corpuscles, was unable to take the curvature which the elasticity of the flagellum would naturally cause.

I give in a table below a number of measurements of ten trypanosomes from standard preparations made in the following way. The trypanosomes were first of all carefully drawn with the camera lucida, projected to a magnification of 3000 linear, as determined by substituting for the trypanosomes a stage-micrometer, of which the divisions were drawn in the same manner and the resulting scale measured. The magnification thus obtained depends, other things being constant, on the height of the camera lucida from the drawing board, that is to say, on the length of the tube of the microscope. By trial a length of tube was found, which, with the combination of lenses used, projected the divisions of the micrometer-scale, each representing ·01 mm., to a scale in which they were 30 mm. apart. Taking the magnification of 3000 obtained in this way as accurate, the measurements shown in the table were obtained.

The measurements were carried out by following the undulations of the trypanosomes in the figures with a piece of thread, the length of which was then measured in millimetres; the figure obtained was divided by three, and the resulting figure represents, therefore, the actual length in microns. The pre-nuclear region is measured from the karyosome, or when two nuclei are present (fig. 6) from the boundary between them, to the extreme anterior end of the body; the free flagellum from the latter point to the end. As it is often difficult to determine exactly the anterior

termination of the body, these two measurements are a little uncertain and subject to considerable variation. The internuclear region is measured from the karyosome to the centre of the kinetonucleus; the post-nuclear length is from the kinetonucleus to the posterior extremity of the body. The undulating membrane is measured by following up the sinuosities of the marginal flagellum from the kinetonucleus to the anterior extremity of the body.

Total length (body and free flagellum).	Body.				Flagellum.	
	Pre-nuclear region.	Inter-nuclear region.	Post-nuclear region.	Greatest breadth.	Undulating membrane.	Free flagellum.
(Fig. 1) 28	—	—	—	1·5	21	7
(Fig. 2) 32	—	—	—	2	24	8
(Fig. 3) 30·5	6	12	4·5	2	21	8
(Fig. 4) 28·6	6	11	4	1·6	27·6	7·6
(Fig. 5) 28	8	11·3	3·3	1·6	21·4	5·6
(Fig. 6) 31	7	12·5	5	1·6	24	7
(Fig. 7) 33	6·3	13	5·3	1·5	25	8·3
(Fig. 8) 32	9	12·5	5	1·5	24·6	5·3
32·3	8	12·5	5	1·5	24	7
30·3	6	13	4	1·6	22·5	7
Average 30·57	7·04	12·22	4·5	1·64	23·51	6·88

Salvin-Moore, Breinl, and Hindle have recently (1908) published an elaborate study of Trypanosoma lewisi. Since they deal chiefly with the multiplicative period, when the trypanosomes vary enormously in size and form, it is difficult to compare their results with mine. Their fig. 1 appears to represent a so-called "adult" trypanosome, in the period when multiplication is past, comparable to the forms with which I am dealing in this memoir. If that is so, I think anyone who has the most elementary acquaintance with T. lewisi will agree with me that their technique has distorted and deformed the creature very greatly. This is not surprising to me, since these authors rely on Flemming's fluid as

a fixative, and I have always found this mixture to give the worst results of any that I have tried as regards the general form of the body (compare my figs. 36–38).

It does not, however, follow that because the external form is distorted the internal structure is necessarily falsified. I have got the sharpest differentiation and clearest pictures of the cytological details in preparations fixed with sublimate mixtures, in which the body as a whole is undoubtedly diminished in size. But I do not think that the technique of Salvin-Moore and Breinl (1907) gives them any right to deny the very obvious trimorphism seen in T. gambiense, for instance, since their preparations, to judge by their illustrations, are obviously inferior to the ordinary osmic-vapour Romanowsky preparations for a study of the form, size, and general structure of the body. I have elsewhere contested their statement that T. gambiense shows no form-differentiation. T. lewisi, on the other hand, is certainly remarkably uniform in structure during the non-multiplicative period. It is not possible to distinguish any distinct types of form, nor anything but slight variations in size, as shown in my table above. The differences that can be observed in the kinetonuclei of different forms are dealt with below.

(2) The Periplast.

That trypanosomes have a fairly strong and resistant membrane or cuticle at the surface of the body is obvious from the manner in which they preserve their body-form under trying circumstances. This fact is brought home to anyone who has studied the trypanosomes and trypanoplasms of fishes. As I have shown elsewhere,[1] in smears of fish-blood dried before fixation the trypanosomes may be almost perfect in form and appearance, while the trypanoplasms side by side with them in the same preparations are deformed almost beyond recognition. This alone is a sure indication that the body-cuticle, commonly termed periplast, is very delicate in the trypanoplasms but comparatively strong in the trypanosomes.

[1] 'Proc. Zool. Soc.,' 1909, pp. 3—31, Pls. I—V.

In Trypanosoma lewisi it is not difficult to discover the existence of a fairly thick periplast, which can be stained and shown up by a variety of methods. After the Romanowsky stain it is usually seen at the edge of the body as a red line, most distinct as a rule on the side opposite to the flagellum, where sometimes it is almost as deeply stained as the flagellum itself (figs. 80, 81). With Twort's stain the periplast stains green and is often very distinct (Pl. 23). With iron-hæmatoxylin the periplast stains very faintly and appears excessively delicate.

In many preparations the periplast becomes very distinct, not by being coloured itself, but by the layer of the body immediately under it becoming clear and transparent, so that the periplast appears, on the side opposite to the flagellum, to stand out from the body as a delicate line, very easily overlooked in preparations in which it is not stained. Figs. 64–69 show very well the genesis of this periplast-line in the process of extraction of Giemsa's stain. It is also seen well in the preparations fixed with Schaudinn's fluid followed by Twort's stain (fig. 85), which colours the periplast green, as already stated.

In preparations stained with Romanowsky stain, after being preserved in various ways, and either dried off or mounted in Canada-balsam without ever being dried, some trypanosomes may be found showing creases and folds of the periplast, which are stained red and simulate fibrils running more or less longitudinally (see especially figs. 70–72). I shall return to this point again later on.

In or immediately under the periplast there are found in many species of trypanosomes, as is well known, distinct contractile fibrils or myonemes. I have myself seen very clearly, and described elsewhere, myonemes in Trypanosoma percæ; less distinctly in Trypanosoma granulosum. I found them most clearly shown in preparations fixed first with osmic vapour, then with Schaudinn's fluid, and stained with iron-hæmatoxylin, very slightly extracted.

In T. lewisi I have not succeeded in seeing myonemes, in spite of much searching of preparations fixed and stained in

various ways. I do not know whether my failure to find them is due to the minuteness of the object, or to my not having hit off just the right degree of extraction of the iron-hæmatoxylin stain. The trypanosomes in which I have seen myonemes have been very large species, and it is possible that in T. lewisi the myonemes are too minute to be resolved with the magnification used. On the other hand, myonemes appear to give up the stain very readily, while if over-stained they are obscured by the darkness of the preparation. There can be hardly any doubt that these active flexible organisms must possess a contractile apparatus which suitable methods of technique or optics would reveal.

(3) The Cytoplasm.

In the thickest part of the body, that is to say, in the internuclear region and immediately in front of the trophonucleus, the cytoplasm shows commonly in preparations two distinct regions, a peripheral zone, situated immediately below the periplast, and an axial portion.

The peripheral zone is usually seen in the ordinary Romanowsky preparations as a more or less distinct border marked off by its red tint from the axial bluish region (figs. 60, 61). When the stain is extracted, however, the peripheral zone becomes clear and apparently empty, leaving the periplast-line standing out in the manner already described (figs. 62–69). It is quite evident, from a comparison of different specimens, especially as regards breadth, that the appearance of the periplast-line is really due to the clearing up of the region immediately below the periplast and not to a dilatation of the periplast, or artificial raising up of it from the body. The question is, How is the clearing up of the region under the periplast effected ? Is it a clear zone of protoplasm which takes the red stain, and from which the stain is extracted, or is it really an empty space in which the stain is deposited and from which it is dissolved out again ? And if it is an empty space is it one naturally present, filled only with fluid in the living condition, or is it produced artificially by shrink-

age and contraction of the body-cytoplasm? These are difficult questions to answer, but I think that, so far as the trypanosomes in the preparations are concerned, the clear zone is shown to be really a space: first, by a comparison of the trypanosomes fixed by certain methods, for example, Mann's fluid (figs. 29–35, 77–79, 86, 87), in which a comparison with other preparations shows clearly that the thinness of the body is due to an obliteration of the clear area under the periplast by shrinkage, so that the periplast comes into contact with the axial portion of the cytoplasm; secondly, from the frequent creases and folds in the periplast already mentioned, indicating the existence of a space under it which is either empty or at least not completely filled out.

Since, on the other hand, no trace of a clearer peripheral zone can be seen in the living trypanosome nor in the standard preparations, I am inclined to think that the appearance of the peripheral clear zone under the periplast is due simply to shrinkage of the cytoplasm in preparations, leaving a space at the periphery in which the red stain of the Romanowsky combination becomes deposited. This conclusion may be supported also on general grounds; it is common in protozoa to find a more fluid endoplasm surrounded by a less fluid ectoplasm. It is, on the other hand, very unusual to find the peripheral region of the cytoplasm of more fluid nature than the region situated more internally.

For all these reasons I am of opinion that the clear space seen under the periplast is an artefact, the result of shrinkage of the cytoplasm produced by the processes of dehydration and clearing, necessary when never-dried preparations are mounted in Canada-balsam. No such space is ever found in dried-off preparations.

Apart from the nuclear and locomotor apparatus, the cytoplasm contains various enclosures. First in importance are certain granules situated chiefly in the inter-nuclear region; as it is convenient to employ a distinctive term for them, I propose to use the term chromatoid grains.[1]

[1] Compare Woodcock, 'Quart. Journ. Micros. Sci.,' vol. 50, p. 229.

These bodies are best seen after Twort's stain, which colours them red in the midst of the greenish cytoplasm (figs. 82–87). They are also well seen after iron-hæmatoxylin if the stain be but little extracted (figs. 28–30), and are most distinct when the trophonucleus appears as an evenly stained black patch without any detail; if, however, the extraction be carried further the stain comes out of them, and in preparations in which the karyosome stands out sharply from the trophonucleus the chromatoid grains are no longer visible (figs. 14, 15, 25, 31, 32). With the Romanowsky stain the chromatoid grains can seldom be made out; sometimes (figs. 61, 74, 80) a few red granules are to be made out, but more usually the cytoplasm stains an even bluish or purplish tint, which may mean either that the chromatoid grains are stained red, but are obscured by the dense blue colour of the general cytoplasm, or that they also take the blue coloration of the Romanowsky stain. After Delafield's hæmatoxylin the chromatoid grains stain a dull violet like the nucleus; after gentian-violet-orange and safranin-gentian-orange they also stain like the nucleus; after methylene-blue-eosin they take the blue colour. With all these stains, however, and also with iron-hæmatoxylin, the general cytoplasm is tinged in the same manner, probably owing to the presence of minute granulations diffused through it which also take up the stain. Hence the chromatoid grains do not appear after the five staining methods mentioned as definite sharply marked bodies, but as blotchy, ill-defined patches which give the cytoplasm a marbled appearance.

Twort's stain alone, of all I have tried, differentiates the chromatoid grains clearly from the surrounding protoplasm. By this method they appear as coarse granules stained a faint reddish tinge, irregular in form and not sharply contoured. They vary in amount, being sometimes spread over the whole inter-nuclear region, and even extending into the pre-nuclear region; in other cases there are only a few of them, occurring chiefly just behind the trophonucleus (figs. 82–87). These variations in the quantity of the chromatoid grains do not

appear to be correlated with any other structural variations of the trypanosome.

By their reactions to the stains mentioned, the chromatoid grains are evidently allied to the chromatin-elements of the nuclei; they are stained in a manner similar to the trophonucleus by hæmatoxylin, gentian-violet, safranin, methylene-blue, and neutral red (in Twort's stain). The Romanowsky stain alone fails to differentiate them clearly, but I have said enough already in support of my opinion that this stain is quite unreliable as a test for nuclear structures. Comparing the chromatoid grains with the constituents of the trophonucleus (see below), it is clear that their reactions agree very closely with the intra-nuclear chromatin. In preparations in which the stain is extracted from the intra-nuclear chromatin, leaving the karyosome sharp, it is also extracted from the chromatoid grains. When the intra-nuclear chromatin is coloured, the chromatoid grains are also coloured, and to about the same tint. On these grounds I infer that the chromatoid grains represent extra-nuclear chromatin or chromidia, derived from the trophonucleus, possibly from the karyosome (see below).

In ordinary preparations the cytoplasm does not contain any other enclosures than the chromatoid grains. In my standard preparations, however, examined in the wet blood, I find a body which is not to be made out in trypanosomes examined in Canada-balsam or cedar oil (figs. 1–8). Quite constantly a refringent granule is seen, sharply and distinctly, in the post-nuclear region, sometimes close behind the kinetonucleus, sometimes nearer the pointed posterior termination of the body. It has the usual appearance of a refringent granule appearing as a black dot at the lower focus and as a clear spot at the higher focus. It is more refringent and much more distinct than the unstained kinetonucleus. At first I thought it might be Prowazek's "anchoring granule," but I do not find anything anchored to it, and it disappears in the stained and mounted preparation. This is probably due to the fact that the granule itself does

not stain, and is only visible by its refringence, and consequently disappears when mounted in refractive media. As to the nature or function of the granule, I am not able to offer any suggestions.

In many trypanosomes in the standard preparations I find a distinct clear space or vacuole immediately in front of the kinetonucleus, varying in size, frequently absent, sometimes double (figs. 5–8). This is perhaps an artefact or post-mortem change, as I have not been able to see it in the living animal, nor does it occur in the ordinary stained preparation.

(4) Locomotor Apparatus.

Under this head are comprised the flagellum with its basal granule or blepharoplast, and the undulating membrane. I have already described above the general configuration of the flagellum and undulating membrane, and shall deal here only with points of minute structure.

The blepharoplast is situated very close to the kinetonucleus, apparently touching it or even overlapping it; that is at least the position which it has in the standard preparations (figs. 1–8) and in all preparations which have been mounted without ever having been dried (compare figs. 62–69). On the other hand, in preparations which have been dried off, either before or after fixation, a considerable interval usually separates the kinetonucleus and blepharoplast (figs. 60, 61, 70, 73, 80, 81), a state of things which must be regarded as artificial. In view of the alleged purity of the technique of Salvin-Moore, Breinl, and Hindle (1908), I am surprised to notice such a condition very frequently in their figures.

In preparations stained with iron-hæmatoxylin or other sharp nuclear stains the blepharoplast appears as a minute granule, a slight dilatation, often hardly visible, of the proximal end of the flagellum (figs. 25–32, etc.). After Twort's stain it is coloured green, like the flagellum itself, in sharp contrast with the nuclei, which are red (figs. 82–87). After the Romanowsky stain the blepharoplast is coloured red, like

the flagellum and the nuclei, and instead of appearing as a very minute dot, it is usually considerably enlarged in size, appearing sometimes as a relatively large, diffuse patch (figs. 70, 73). I regard this appearance of the blepharoplast simply as another instance of the tendency of the Romanowsky stain to form concretions, as it were, round the bodies for which has the affinity, whatever it may be, which is shown by staining.

The flagellum presents itself as a delicate filament of even thickness and like appearance throughout its whole length (figs. 10, 11, etc). I have not been able to see any structural differences in it in different parts. After the Romanowsky stain the flagellum commonly appears much thicker and quite coarse in structure, a state of things evidently due to the usual performances of this staining method. When, however, the Romanowsky stain is suitably extracted, the flagellum comes down to its normal thickness (figs. 67–69).

Prowazek (1905) has introduced a most extraordinary complication of fibrils into the structure of T. lewisi (see his text-fig. 2B, p. 359). One fibril is supposed to connect the karyosome with the kinetonucleus; from the latter another fibril runs to an "anchoring grain" situated in the post-nuclear region, and from this grain yet another fibril runs forward to the anterior end of the body. I have searched in vain in all my preparations, without finding a trace of this fibrillar system. In my smears stained with the Romanowsky stain, however, I find that the periplast, stained red, shows creases and folds often closely simulating distinct fibrils. I have represented some of these in figs. 70–72 (Pl. 22), and could have drawn many more. I have a strong suspicion that Prowazek's fibrillar system is founded on nothing but accidental creases of this kind, since he remarks, "Die Fasern selbst lassen sich färberisch nur schwer darstellen und nur an weniger Objekten konnte man sie streckenweise als rote Fibrillen verfolgen" (loc. cit., p. 358). This so exactly describes what I have seen that I must express my profound scepticism as to the existence of Prowazek's fibrillar system.

(5) The Nuclear Apparatus.

Under this head I include simply the two nuclei, trophonucleus and kinetonucleus. I will begin my account with the second of these two bodies.

The kinetonucleus is easily made out in the living condition or in the standard preparations as a refringent body of fair size a short way from the hinder end. It is also seen, especially in the fixed preparations, that there are two types of form presented by the kinetonucleus, which may be either rod-shaped or circular in contour. Transitions occur, but not commonly, between these two types.

With iron-hæmatoxylin the kinetonucleus stains black; the rod-like type stains much more darkly than the round form, which appears of a pale greyish tint when the stain is well extracted; it is then not so dark in tint as the blepharoplast, which is seen as a black dot on the edge of the kinetonucleus (figs. 31, 33). The rod-shaped type of kinetonucleus, on the other hand, takes the stain more darkly than the blepharoplast which is seen close beside it, not overlapping it (figs. 32, 34).

By all nuclear stains the kinetonucleus is stained intensely, more so than any other part of the body, in fact it may be the only part of the body visible in the preparation. After methylene-blue-eosin it is blue; after Twort's stain, red; after the Romanowsky stain, as is well known, it stains a deep purple-red; what is not so generally known is that it comes out, as I have already pointed out, four or five times its natural size (compare figs. 62, 63, with figs. 10, 11). When, however, the Romanowsky stain is suitably extracted, the kinetonucleus comes down to the size that it appears in other preparations (figs. 66, 69, etc.).

I have not been able to make out any structure in the kinetonucleus. It appears to consist simply of a dense mass of chromatin, which is carried on, or impregnates, a basal substance, for which the name "plastin" is in common use.

With regard to the two forms of the kinetonucleus, it is possible that these may be simply two views of the same

thing, a body having the form of a disc which, seen edgeways, would appear rod-shaped, and in surface view would present a circular contour. This explanation is compatible with the facts stated above, namely that the rod-shaped type is darker in colour, that there are transitions between the two forms, and that the blepharoplast overlaps the round form, but is found close beside the rod-shaped form. I have not been able to convince myself that this explanation is either true or false.

Salvin-Moore, Breinl, and Hindle (1908) are of opinion that the kinetonucleus is to be regarded as of the nature of a centrosome or blepharoplast, and use the term "end-bead of the flagellum" for the granule termed by me the blepharoplast. The question is one that must be decided by developmental data; a true centrosome is pre-eminently a body of dynamic rather than static function. I do not desire, therefore, to argue the question in this memoir, in which I purposely avoid dealing with division-stages. I will only say that in refusing to allow the kinetonucleus the status of a true nucleus, the authors are ignoring a great deal of work by Schaudinn and others in support of this view. As regards the "end-bead," I am still of opinion that it represents a true centrosome or blepharoplast. In Trypanosoma grayi I found that division of the blepharoplast was invariably the first step in the division of the trypanosomes.

The trophonucleus is at first very puzzling, on account of the extraordinary difference in its appearance after the Romanowsky stain, now so much in use, and all other stains. The coarse "Romanowsky splotch" seems at first sight to have nothing in common with the delicate, refined structure seen after iron-hæmatoxylin or Twort's stain (compare figs. 60–63, with figs 10, 11, and 82–87). I have already attempted above an explanation of the vagaries of the Romanowsky stain; my reasons for regarding the results of this stain as departures from the truth are simply founded on the facts—first, that the condition seen after iron-hæmatoxylin is also seen in the standard preparations, in the living condition, and after every other nuclear stain known to be of value as such;

secondly, that when the Romanowsky stain is cautiously extracted the nucleus comes down to a condition similar to that seen after other stains (figs. 68, 69).

The parts of the trophonucleus may be considered under the heads of the "membrane," the "karyosome" or "karyosomes," and the "intra-nuclear chromatin," the latter term being understood to be exclusive of the karyosome.

The membrane appears as a faint but distinct line circumscribing the oval contour of the nucleus. It does not, however, stand out sharply from the contents, nor does it stain a different colour from the rest of the nucleus; it also never shows a double contour. For all these reasons the membrane may be regarded simply as the superficial limit of the intra-nuclear chromatin, condensed to form a more closely knit zone at the surface. In other words, the membrane is not a true nuclear membrane in the sense in which the term is used for Metazoan nuclei.

The karyosome appears usually as a single small grain at or near the centre of the nucleus; this is the commonest condition, found in about 80 to 90 per cent. of the trypanosomes. I regard this, therefore, as the normal state of the nucleus, and will deal with it first, and describe afterwards other conditions. In order to see the karyosome clearly it is necessary to extract the stain thoroughly when staining with iron-hæmatoxylin, otherwise the karyosome is obscured by the granules of the intra-nuclear chromatin. The extraction should be carried on until the karyosome appears as a sharp, clear, and definite body. With Twort's stain no such precautions are necessary, as the intra-nuclear chromatin does not take it up to the same extent, hence this stain is one of the best for showing the karyosome and the nuclear structure generally. In its reactions the karyosome is very similar to the kinetonucleus, but stains less deeply and gives up the stain more readily. I infer from this that the karyosome is, like the kinetonucleus, a body consisting chiefly of chromatin on a basis of plastin, but that the chromatin is much less concentrated and condensed in the karyosome.

The karyosome shows very considerable variations in some of the nuclei. In the first place it varies in size, being usually quite a minute granule, but sometimes very much larger. In the second place it varies in position, and instead of being central or sub-central it may be very excentric in position, even to the extent of being placed quite at one pole of the oval nucleus. Thirdly, the karyosome, usually single, may become double or multiple, apparently through a process of division or disruption. I have described elsewhere[1] such a process of budding on the part of the karyosome in the case of Trypanosoma granulosum, where the large size of the object makes the process easy to follow out in detail. The minuteness of T. lewisi makes it much more difficult to be quite certain what exactly takes place. Appearances can be found, however, which indicate clearly a process of division or budding from the karyosome (fig. 50), leading to the formation of two karyosomes, subequal or markedly unequal in size; the two bodies thus formed travel apart, and may place themselves at opposite poles of the nucleus (fig. 49). One or both of the karyosomes may undergo further disruption, apparently, so that in addition to a principal karyosome there are smaller granules present, not more than four in number in any case that I have observed (figs. 52, 53, 90); I feel, however, considerable doubt whether some of these smaller granules may not be, in most cases, granules of the intra-nuclear chromatin from which the stain has been insufficiently extracted. In my preparations stained with Twort's stain I find deviations from the condition with a single sub-central karyosome excessively rare; I have, however, seen in these preparations the condition with a double karyosome (fig. 86), and with a larger and two smaller karyosomes (fig. 90).

Prowazek (1905, pp. 361–363) has sought to bring the various conditions of the karyosome under three processes, which he terms "autosynthesis," "reduction," and "parthenogenesis." In autosynthesis the karyosome is supposed

[1] 'Proc. Zool. Soc.,' 1909, pp. 21, 22, pl. V, figs. 78–93.

to divide into two, and each half divides again; two of the four parts are thrown out, the other two parts "copulate," and the nucleus is reconstituted. Equally complicated processes are seen in reduction and parthenogenesis.

For my part I am very doubtful if the different appearances seen in the nuclei of T. lewisi will bear the excessive weight of subjective interpretation which Prowazek places upon them. Moreover, Prowazek seems to have based all this theoretical superstructure upon Romanowsky-stained preparations, which, in my opinion, are altogether false and misleading for minute nuclear structure.

All that can be inferred from a comparison of different trypanosomes is that the karyosome sometimes gives off smaller portions; some of these go to the surface of the nucleus, and I think it highly probable that the chromatoid grains described above take origin in this way.

The intra-nuclear chromatin shows different conditions according to the stain used and the degree of extraction of the stain. With iron-hæmatoxylin, for instance, the whole nucleus is at first a dense black patch, owing to the intra-nuclear chromatin being stained so deeply as to obscure completely the karyosome. A similar condition is seen after the Romanowsky stain. As the stain is extracted, the intra-nuclear chromatin gradually becomes lighter in tint, and the karyosome begins to stand out. The extraction of the colour from the intra-nuclear chromatin does not take place uniformly, however, but some of the granules retain the stain longer than others, while some parts become quickly decolorised; there is very often a distinct clear halo round the karyosome (figs. 17, 18, 39). With complete extraction the intra-nuclear substance becomes pale and gives the impression of an empty space, in which the karyosome is suspended (fig. 10, 11). A quite similar and parallel series of appearances is seen when the Romanowsky stain is slowly extracted, as described above, but in this case the granules in the intra-nuclear space are much larger, more of the nature of concretions, than they are after iron-hæmatoxylin. With Twort's

stain the intra-nuclear substance appears a pale red tint, and does not show granulations (Pl. 23); with methylene-blue-eosin it is pale blue. In the standard preparations stained with methyl-green no granulations are to be made out in the nucleus, which has simply the appearance of an oval vesicle, in which the distinct karyosome is suspended (figs. 1–8).

From all these various appearances I conclude that the intra-nuclear space contains, probably, in the living condition a more or less fluid nuclear sap, in which chromatin is suspended in the form of particles, perhaps differing in size or in concentration of substance, but for the most part too minute to be resolved with the microscope, except when very deeply stained, or artificially enlarged by the deposit of the stain round them.

In the foregoing paragraphs I have dealt with the minute structure of the trophonucleus; I will now mention some of its variations as a whole. In the first place it varies considerably in size, as may be seen from my figures. There may sometimes, moreover, be two trophonuclei present, flattened against each other and each of relatively small size (figs. 6, 84). This condition, which is found in a very small number of the trypanosomes, does not appear to have anything whatever to do with division, as all other parts of the body are in the normal single condition; I regard it simply as an abnormality. In rare cases trypanosomes are met with having three trophonuclei (fig. 74).

In preparations fixed with Flemming's fluid (figs. 36–38) and Hermann's fluid (figs. 40–44) the trophonucleus often seems to float in a space in the cytoplasm, a condition which must be regarded as an artefact.

In this memoir I have only dealt with the structure of Trypanosoma lewisi in the resting, "adult" stage. It is my hope in a future memoir to extend these studies to the multiplication-period, and possibly to other developmental periods of the life-history.

Lister Institute,
January, 1909.

List of References.

Prowazek, S. v.—"Studien über Säugetier-Trypanosomen," i, 'Arb. k. Gesundheitsamte,' xxii, pp. 351–395, pls. i–vi, four text-figs. (1905).

Salvin-Moore, J. E., and Breinl, A.—"The Cytology of the Trypanosomes," 'Ann. Trop. Med. Parasitol.,' i, pp. 441–480, pls. xxxviii–xlii (1907).

——— ——— and Hindle, E.—"The Life-History of Trypanosoma lewisi," ibid., ii, pp. 197–220, pls. ii–v (1908).

Schaudinn, F.—"Studien über Krankheitserregende Protozoen: ii, Plasmodium vivax," 'Arb. k. Gesundheitsamte,' xix, pp. 169–250, pls. iv–vi (1902).

——— "Generations- und Wirtswechsel bei Trypanosoma und Spirochæte," ibid., xx, pp. 387–439, twenty text-figs. (1904).

EXPLANATION OF PLATES 21–23,

Illustrating Mr. E. A. Minchin's paper on "The Structure of Trypanosoma lewisi in Relation to Microscopical Technique."

All the figures represent preparations of Trypanosoma lewisi, drawn with the camera lucida at a magnification of 3000 diameters (see p. 760).

In order to facilitate the comparison of the results obtained by different methods, the figures may be best classified by the dates on which the trypanosomes represented were preserved. All those of a given date were from the same blood, preserved or stained by various methods.

February 24th.—Pl. 21, figs. 14, 15, 19–22, 39; Pl. 22, figs. 60, 61, 70, 73, 80, 81). (The preparations of this date were made on slides; all others, except fig. 74, were made on coverslips.)

March 5th.—Pl. 21, figs. 9–13, 36, 37; Pl. 22, figs. 62–69.

March 16th.—Pl. 21, figs. 23, 24, 40–53.

March 20th.—Pl. 21, figs. 25–34, 54–59; Pl. 22, figs. 76–78.

March 27th.—Pl. 21, figs. 17, 18, 38; Pl. 22, fig. 75.

March 30th.—Pl. 23, figs. 82–84, 88–91.

April 3rd.—Pl. 23, figs. 85–87.

April 6th.—Pl. 21, figs. 1–8, 16, 35; Pl. 22, figs. 71, 72, 79.

The following is a classification of the figures according to the method of fixation employed for the preparations :

Osmic vapour simply : Pl. 21, figs. 1–8.

Osmic vapour followed by absolute alcohol : Pl. 21, figs. 9–11 ; Pl. 22, figs. 60–69, 70, 71, 72, 74.

Osmic vapour followed by sublimate-acetic (95 : 5) : Pl. 21, figs. 14–18 ; Pl. 22, figs. 70, 73, 75.

Osmic vapour followed by Schaudinn's fluid : Pl. 21, figs. 23, 24, 45–53.

Osmic vapour followed by Flemming's fluid : Pl. 21, fig. 39 ; Pl. 22, figs. 80, 81.

Sublimate-acetic (99 : 1) : Pl. 21, figs. 12, 13.

Sublimate-acetic (95 : 5) : Pl. 21, figs. 19–22 ; Pl. 23, figs. 82–84, 88–91.

Schaudinn's fluid : Pl. 21, figs. 25–28 ; Pl. 22, fig. 76 ; Pl. 23, fig. 85.

Mann's fluid without formol : Pl. 21, figs. 29–32 ; Pl. 23, fig. 86.

Mann's fluid with formol : Pl. 21, figs. 33–35, 54–59 ; Pl. 22, figs. 77–79 ; Pl. 23, fig. 87.

Flemming's fluid : Pl. 21, figs. 36–38.

Hermann's fluid : Pl. 21, figs. 40–44.

Figs. 19–22, 39, 60, 61, 70, 73, 74, 80, and 81 are from preparations dried off after fixation or after staining ; all others are from never-dried preparations.

In studying the preparations, at least three trypanosomes were drawn from each, in order to take the average of the individual variations, which, though slight, must still be reckoned with, as seen from figs. 1–8 and the table on p. 789. It was, however, neither possible nor desirable to reproduce all these figures. Where only one or two figures are given from a preparation it must be understood that they represent, as far as possible, the average proportions of the trypanosomes in the preparation. Hence such figures as 21, 22, 75, 79, etc., are not to be taken as representing trypanosomes of small size, but as normal individuals showing the shrinkage produced by the method of preparation, and should be compared with others of the same date (see above) prepared in other ways.

PLATE 21.

Figs. 1–8.—" Standard " preparations (see p. 759). 1 and 2 fixed with osmic vapour in a hanging drop ; owing to the thickness of the preparation details could not be made out, but only the outline of the body and the flagellum could be drawn. 3 and 4. Fixed with osmic vapour in a hanging drop, then mounted on a slide and sealed up ; the trophonucleus with its karyosome, kinetonucleus, and posterior refringent granule are seen. 3 has a rod-shaped, 4 a rounded kinetonucleus.

5–8. Treated as 3 and 4, but with the addition of acidulated methyl-green solution to the preparation. The same details are seen as in the foregoing, with the addition of a vacuole or vacuoles immediately in front of the kinetonucleus, probably to be regarded as artefacts.

With figs. 1–8 compare especially figs. 16, 35, 71, 72, 79, all drawn from preparations made from the same blood at the same time, but preserved in different ways.

Fig. 9.—Outline of a specimen from the same preparation as figs. 62, 63, showing the flagellum passing on the concave side of a body-curve at a point near its origin, and here having a sharp double bend.

Figs. 10, 11.—Osmic vapour, absolute alcohol, iron-hæmatoxylin, never-dried. Note the distinct periplast-line. Compare especially with figs. 12, 13, 36, 37, 62–69, all preserved at the same time and from the same blood.

Figs. 12, 13.—Sublimate acetic (99 : 1); 12, stained Giemsa; 13, stained iron-hæmatoxylin; never-dried. Compare with figs. 10, 11, etc.

Figs. 14, 15.—Osmic vapour followed by sublimate-acetic (95 : 5), stained iron-hæmatoxylin; never-dried. Compare figs. 19–22, 39, 60, 61, 70, 73, 80, 81, all preserved at the same time and from the same blood.

Fig. 16.—Fixation as in the last; stained Giemsa; never-dried. Drawn in outline to show the great diminution of size. Compare figs. 1–8, etc., made from the same blood at the same time. All the trypanosomes in the same preparation and in another treated in the same way at the same time show the same reduction in size.

Figs. 17, 18.—Fixation as in 14–16; stained iron-hæmatoxylin; never-dried. Compare with figs. 38 and 75, both preserved at the same time from the same blood.

Figs. 19–22.—Fixed with sublimate-acetic (95 : 5) direct. 19, 20. Stained iron-hæmatoxylin; never-dried. 21, 22. Stained Giemsa, then dried off, showing extraordinary shrinkage. Compare figs. 14, 15, etc.

Figs. 23, 24.—Osmic vapour, followed by Schaudinn's fluid; stained iron-hæmatoxylin; never-dried. Note the very distinct periplast-line. Compare figs. 40–44. Preserved at the same time from the same blood.

Figs. 25–28.—Fixed with Schaudinn's fluid direct; stained iron-hæmatoxylin; never-dried. 25, much extracted; 26, 27, less; 28, still less extracted. Compare figs. 29–34, 76–78, preserved at the same time from the same blood.

Figs. 29–32.—Fixed with Mann's picrocorrosive without formol; stained iron-hæmatoxylin; never-dried. 29, 30, less, 31, 32, more extracted. Compare figs. 25–28, etc.

Figs. 33, 34.—Fixed with Mann's picrocorrosive with formol; stained iron-hæmatoxylin; moderately extracted; never-dried. Two trypanosomes in the same field showing the relations of kinetonucleus, blepharoplast, and flagellum very sharply. Compare figs. 25–28, etc.

Fig. 35.—Treated as last. Rather more extracted. Compare figs. 1–8, etc.

Figs. 36–38.—Fixed in Flemming's fluid direct; stained iron-hæmatoxylin; never dried. 36, 37, compare with figs. 10, 11, etc. 38, compare with figs. 17, 18, etc.

Fig. 39.—Fixed with osmic vapour followed by Flemming's fluid; stained iron-hæmatoxylin; never-dried. Compare with figs. 14, 15, etc.

Figs. 40–44.—Fixed in Hermann's fluid direct; 40, stained gentian violet and orange; 41, 42, stained safranin, gentian violet, orange; 43, 44, stained iron-hæmatoxylin; never-dried. Compare figs. 23, 24, etc.

Figs. 45–53.—Nuclei of trypanosomes from the same preparation as figs. 23, 24. 45 shows the most usual condition, occurring in about 90 per cent. of the trypanosomes.

Figs. 54–59.—Nuclei of trypanosomes from a companion preparation to that from which figs. 33, 34 were drawn; treated in the same manner, but the stain rather more extracted. 54 represents the most usual condition.

PLATE 22.

All figures drawn from preparations stained with the Romanowsky stain, either Giemsa's method (G.) or azure-erythrosin (A. E.).

Figs. 60, 61.—Fixed with osmic vapour, then dried off; fixed with absolute (G.). Compare with figs. 14, 15, etc.

Figs. 62, 63.—Osmic vapour; absolute alcohol; never-dried (G.). Compare with figs. 10, 11, etc.

Figs. 64–69.—From a companion slide to the foregoing; more extracted with acetone (see pp. 775–777).

Figs. 70–72.—Trypanosomes showing folds of the deeply-stained periplast simulating fibrils (see p. 797). 70, fixed osmic vapour, then dried off; sublimate acetic (95 : 5), stained (G.); from a companion preparation to that from which fig. 73 is drawn. 71, 72, osmic vapour, absolute alcohol (G.); never-dried. Compare figs. 1–8, etc.

Fig. 73.—Osmic vapour, sublimate-acetic (95 : 5), stained (G.); dried off after the osmic vapour. Compare figs. 14, 15, etc.

Fig. 74.—Trypanosome with three trophonuclei; osmic; absolute alcohol (G.); dried off.

Fig. 75.—Osmic vapour, sublimate-acetic (A. E.); never-dried. Compare figs. 17, 18, etc.

Fig. 76.—Schaudinn's fluid direct (A. E.); never dried. Compare figs. 25–34, etc.

Figs. 77, 78.—Mann's picrocorrosive with formol (A. E.); never-dried. Companion preparation to the last.

Fig. 79.—Treatment similar to the last. Compare figs. 1–8, etc.

Figs. 80, 81.—Osmic vapour, Flemming's fluid (G.); dried off. Compare figs. 14, 15, etc.

PLATE 23.

All figures drawn from preparations stained with Twort's stain; never-dried.

Figs. 82–84.—Fixed in sublimate-acetic (95 : 5).

Fig. 85.—Fixed in Schaudinn's fluid.

Fig. 86.—Fixed in Mann's picrocorrosive without formol.

Fig. 87.—Fixed in Mann's picrocorrosive with formol.

Figs. 88–91.—Nuclei of trypanosomes from the same preparation as figs. 82–84.