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THE MORPHOLOGY OF *TRYPANOSOMA GAMBIENSE* AND *TRYPANOSOMA RHODESIENSE* IN CULTURES : AND A COMPARISON WITH THE DEVELOPMENTAL FORMS DESCRIBED IN *GLOSSINA PALPALIS*

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I. INTRODUCTION

This research was undertaken at the suggestion of Sir Ronald Ross under funds allotted by Sir Edwin Durning-Lawrence (Bart.), in the case of one of us (J.G.T.), and under a grant from the Post-

Graduate Research Fund of the Queen's University of Belfast in the case of the other (J.A.S.). We wish to acknowledge our indebtedness to Sir Edwin Durning-Lawrence, Bart., and to the Queen's University of Belfast for their generous help, and to thank Sir Ronald Ross, Dr. J. W. W. Stephens, and Dr. H. B. Fantham for their kind interest and the valuable aid given to us.

The results recorded in this paper have been obtained from experiments conducted in the Liverpool School of Tropical Medicine during a period of over six months. We have directed our attention to the cultivation of trypanosomes pathogenic to man, namely, *Trypanosoma gambiense* (Dutton) and *Trypanosoma rhodesiense* (Stephens and Fantham).

The first experiments were made only with *Trypanosoma rhodesiense*, and a preliminary note by one of us (J.G.T.) was published in the Annals of Tropical Medicine and Parasitology, in May, 1912, giving a short account of the cultivation of this trypanosome.

It was noted that this trypanosome in culture assumed morphological characters which strongly suggested that we were obtaining in culture tubes a development similar to that which takes place in the intestinal tract of tsetse flies. On this account it was decided to attempt the cultivation of *T. gambiense* in order to compare its cultural morphology with the developmental forms of this trypanosome found by Sir David Bruce and his colleagues (Captains Hamerton, Bateman and Mackie) (1911 b) in *Glossina palpalis*. Unfortunately there is at present no description of the developmental characters of *T. rhodesiense* in the tsetse-fly, so that in this paper it is only possible to compare the cultural characters of this trypanosome with those of *T. gambiense*. We await, therefore, with great interest, a description of the morphology of *T. rhodesiense* in the fly, so that a comparison may be made with the forms found in culture.

II. HISTORICAL

Numerous attempts have been made, with more or less success, to cultivate pathogenic trypanosomes ever since MacNeal and Novy (1903) found that it was possible to cultivate *T. lewisi*, for apparently an indefinite period, on an artificial medium composed of a mixture

of nutrient agar and defibrinated blood. Novy and MacNeal (1903-1904) succeeded in cultivating *T. brucei*, but their results were not so successful as in the case of *T. lewisi*, although they managed to get sub-cultures and to infect animals from these. The cultural characters of this trypanosome were found by them to differ essentially from those of *T. lewisi*.

Novy, MacNeal and Hare (1904) were also successful in cultivating *T. evansi*, but were unable to obtain sub-cultures, and inoculation experiments gave negative results. The results of Novy and MacNeal with *T. lewisi* and *T. brucei* were confirmed by Smedley (1905) in most of the important details, but this observer was unable to cause infection by inoculating cultures of *T. brucei*.

The first record of any attempt to cultivate the trypanosomes pathogenic to man was that of Thomas and Breinl (1905). These observers succeeded in keeping *T. gambiense* alive (' by transference from tube to tube ') for sixty-eight days on a blood agar medium composed of blood and nutrient agar made up with veal or chicken meat infusion. In the next year Gray and Tulloch (1906) using a blood agar medium made with dog's blood which had not been defibrinated, succeeded in keeping cultures of *T. gambiense* alive until the twentieth day, when the cultures were overrun by a growth of cocci. Sub-cultures were unsuccessful. These observers noted the resemblance of the cultural forms to those found in the tsetse-fly after feeding on animals infected with *T. gambiense*. They found on the fifteenth day very large forms sometimes 54μ in length. Laveran and Mesnil attempted to cultivate *T. gambiense* in blood agar tubes, and found motile trypanosomes 35 to 40 μ long twenty days after.

Various other trypanosomes have been successfully cultivated, and an excellent epitome of this subject will be found in the Sleeping Sickness Bulletin (1909).

III. TECHNIQUE

(*a*) *Culture Media.* Many experiments have been made during this investigation in the endeavour to obtain a suitable medium. Fluid media such as nutrient broth and horse serum to which haemoglobin had been added proved unsuccessful. The following fluid medium, however, was found to be of great use. Human pleuritic fluid

was rendered complement free by heating to 45°C. for one hour. This was added to small sterile test tubes, about 1 c.c. to each tube, and into each was introduced about one-third volume of citrated human blood. These were then placed in a water bath at 45°C. for half an hour. In this culture fluid it was found that both *T. rhodesiense* and *T. gambiense* developed, and in one tube we found *T. rhodesiense* continued to exist for twenty days at room temperature. This is most interesting when we take into consideration the fact that we were experimenting with human blood, and the trypanosomes used were those pathogenic to man. From this medium sub-cultures were successfully obtained in the modified Novy-MacNeal-Nicolle medium described below.

In a preliminary note on the cultivation of *T. rhodesiense* (1912), it was announced that this trypanosome had been grown on a modification of the Novy-MacNeal-Nicolle medium (Nicolle, 1908), made by substituting for defibrinated rabbit's blood, citrated rat's blood heated to 45°C. for half an hour, and by using sea salt for ordinary sodium chloride. Since writing this, however, it has been found that pure sodium chloride is probably just as efficient in making up the medium. Most of our experiments have been made with this modification of the Novy-MacNeal-Nicolle medium, and we have so far found it the most suitable for cultures and sub-cultures of both *T. rhodesiense* and *T. gambiense*. A full description of the technique used in its preparation is given below. The agar medium is made up as follows:—Agar, 14 grams; sodium chloride (pure), 6 grams; and distilled water, 900 c.c. The agar must previously be washed thoroughly to remove, as far as possible, all impurities. This is done by allowing it to soak for about thirty-six hours in distilled water, which should be frequently changed. This agar medium was then added in quantities of 3 c.c. to sterile test tubes, and sterilised by heating to 100° C. for twenty minutes on three successive days.

A sterile mixture of rat's blood and sodium citrate solution, 1%, is now prepared by the following method. Into a sterile syringe draw up 2 c.c. of a 1% solution of sodium citrate, and then kill a normal rat. Sear the surface of the right ventricle, insert the needle of the syringe into the heart and draw up the blood into the syringe. About 4 c.c. of blood may be procured from a small rat, but of

course the amount varies according to the size of the animal used. Thus a mixture of rat's blood and 1 % sodium citrate solution in a proportion of about 2 to 1 is obtained. The melted agar is cooled to a temperature of 45°C., and at least an equal quantity of citrated blood is added to each tube. Here it is of interest to note that Novy and MacNeal (1904) when cultivating *T. brucei* also found that it was necessary to add at least an equal quantity of blood to the agar in order to obtain a successful culture. These tubes are kept in a water bath at a temperature of 45°C. for half an hour, in order, as far as possible, to destroy the complement without making any appreciable change in the character of the blood. The tubes are sloped, and when cool are protected with rubber caps to prevent evaporation. They are then placed in an incubator at 25°C. for two days to allow the water of condensation to collect, and also to permit of any contamination being observed. In our experience this has proved the most satisfactory medium, but we find that citrated human blood treated in the same way is also good. The fact that in our experiments citrated blood was more satisfactory than defibrinated blood is of interest because Plimmer and Bradford (1899) found that the addition of sodium citrate solution to blood containing *T. brucei* prolonged the vitality of that trypanosome, and also because Rogers (1904) was successful in growing *Leishmania donovani* in citrate solution.

Since experimenting with these media, Dr. Row, of Bombay, has very kindly sent to us a paper (1912) on the preparation of a fluid medium for the cultivation of Protozoa, and we think it might be most suitable for the growing of trypanosomes, but so far we have been unable, through lack of time, to give it a proper trial.

It seems that one of the essential factors for the development of trypanosomes is the presence of a certain amount of fresh free haemoglobin, and under such conditions it is natural that forms similar to those found in the gut of the tsetse-fly will develop, because whenever a fly sucks blood the process of digestion soon breaks up the corpuscles, thus setting free the haemoglobin necessary for the further development of the trypanosomes.

Sir David Bruce and his colleagues (1909b) found that about 5% to 8% of *Glossina palpalis* became infective after feeding on a case of sleeping sickness, and suggested that this was comparable

to that which occurs in cultivation where only a few tubes owing to some unknown reason develop. Novy and MacNeal (1904) in growing *T. brucei* failed twenty-five times in succession to get cultures, and in another series of experiments only four out of twenty-five attempts were successful. We have found the same difficulties in our experiments, and many tubes have been inoculated unsuccessfully without any apparent reason. This difficulty of always obtaining a culture shows that it is necessary to inoculate a large number of tubes in order that at least one or two successful growths may be obtained, and the same applies to sub-cultures which should be made in batches. If this is not done we are almost certain, except when very lucky, to have disappointments, and this uncertainty of obtaining a culture shows that a medium might easily be rejected as being of no use simply because a sufficient number of trials had not been made. The same difficulty makes it uncertain what the best formula for a medium really is, but we think that the modification of Novy-MacNeal-Nicolle medium described in this paper, if carefully tried, will be found to be successful. Strict asepsis must be observed in all cases, because it was found if the cultures became contaminated the trypanosomes died out very rapidly.

(*b*) *Inoculation of media.* The blood used to inoculate tubes was obtained, with aseptic precautions, from the heart of an infected animal which had just been killed. We find, like Smedley (1905), that a rat is the most suitable animal from which to obtain this blood. It is advisable not to add too much blood, and it seems also best to inoculate trypanosomes when there are many of the stout forms present in the blood. It is most difficult, however, to make any definite statement regarding the most suitable time to inoculate from an animal. At first it was thought that it was best to inoculate the tubes early during an infection and so introduce young trypanosomes, but we have found since then that trypanosomes inoculated later, after infection had lasted for a considerable period, also developed well in culture tubes. After many experiments we have concluded that only about three or four drops of blood ought to be added to 1 c.c. of condensation water, and we also think that probably the number of stout forms present in the blood plays an important rôle in development. This may or or may not be the case, since it is so difficult to exclude the uncertainty of the medium.

(*c*) *Incubation of cultures.* Our cultures were usually incubated at a temperature of 22°C. to 24°C. It was found that higher temperatures make the trypanosomes develop more rapidly, but on the other hand they more quickly disappear from the culture fluid. Cultures grown at a temperature of 28°C., like those described in the preliminary note on *T. rhodesiense* (1912), only showed flagellates for seven days. This rapid growth is a disadvantage in that it does not admit of sufficient time to make complete observations of the cultures. The effect of temperature on the cultures has been pointed out by Novy and MacNeal (1904), who found that the higher temperatures not only produced a more rapid developmental change in the trypanosomes themselves, but also more quickly altered the medium. Roubaud (1909) stated that a temperature of 28°C. caused *T. gambiense* to disappear more rapidly from the gut of tsetse flies than lower temperatures did.

(*d*) *Sub-cultures.* Two or three loopfuls from a successful culture are transferred to a new tube by means of a sterile platinum wire, and in a culture grown at a temperature of 22°C. to 24°C. it is found that the sixth or seventh day is usually the most suitable for making such an inoculation. Novy and MacNeal (1904) in cultivating *T. brucei* also found this to be the most suitable time. The success of sub-cultures made at this time appears to be due to the predominance of 'healthy developing forms.'

(*e*) *Examination of cultures. Fresh specimens.* The cultures were examined in fresh preparations every twenty-four hours, and we also kept culture fluid under observation for twenty-four hours by using a hollow slide and cover-glass sealed with vaseline. By these methods the cultures were examined when the trypanosomes were alive, and thus movements, division, and other phenomena could be carefully observed.

Stained specimens. These cultures were most difficult to stain on account of salts and free haemoglobin, and most disappointing results were often obtained, but the following method carried out carefully gives very good slides. To a perfectly clean slide convey a platinum loop full of culture fluid. Shake up the culture fluid thoroughly before doing so. Expose this drop to the vapour of a 4% osmic acid for about 30 seconds, and then with the edge of a piece of cigarette paper make a smear. Expose this smear again

to the osmic acid vapour for a few seconds, and then fix the slide in pure methyl alcohol for twenty minutes. Dry, using no heat Now take a piece of cigarette paper, cover the film with it, and carefully pour on it some Giemsa stain (1 drop to 1 c.c. of distilled water). Allow this to remain on the slide for two or three hours, the cigarette paper acting as a filter and preventing deposit. Wash the slide thoroughly in tap water, and examine before drying under the low and high powers of the microscope so as to determine the depth of staining, which may be too faint. If the stain is too faint pour on some fresh Giemsa for five or ten minutes, and then again wash thoroughly. It is important that the stain used has not deteriorated as so often happens with Giemsa stain if kept for any length of time. If it should happen that the slide is badly stained and looks too blue, it may be restained successfully by decolorising with methyl alcohol and staining several hours film downwards in Giemsa.

IV. CULTURAL CHARACTERS

(a) *Morphology of T. gambiense and T. rhodesiense in Cultures.* As the behaviour of these two species of trypanosomes in cultures is very similar, it is proposed to discuss their cultural characters together and afterwards to describe any points of difference we have observed in these characters. After a period of twenty-four hours it is found on examining some of the culture fluid that many of the trypanosomes have become motionless, many have their flagella torn off, and many are rounded up and disintegrated (Pl. XX, fig. 36). In stained specimens there is also evidence of the death of many of the trypanosomes as is shown by the fact that they may be found in all stages of disintegration. In some the nucleus is broken up, in others it is altogether absent. Many stain badly and are filled with vacuoles. However, in fresh specimens very active trypanosomes are still found, the majority of which appear to be of the 'stumpy' type and average about 20 μ in length (Pl. XIX, fig. 3.) These forms in stained preparations have well defined and well stained nuclei, and a short free flagellum ; the protoplasm stains a dark blue and may contain a few fine chromatin granules. Bruce (1911b) figures stout forms during the first few days after the ingestion of *T. gambiense* by *G. palpalis*. The survival of these stout forms was also found by

Roubaud (1909) and Kleine and Taute (1911) in the gut of
G. palpalis fed on animals infected with T. gambiense.
Swellengrebel (1911) also states that the stout forms are more
resistant to unfavourable influences than the more slender forms in
the blood of mice which had died from an infection of T. gambiense.
This observer suggests that very probably the thick forms found
in the gut of G. palpalis and the blood of dead mice are the more
resistant, and that the predominance of these forms at the beginning
of the development in the fly can be thus explained. From this it
would appear that the stout forms found in the blood might possibly
be forms specially adapted for the further development in the fly, and
this may explain the reason why we have found that cultures were
more successful if made when the stout forms found in the blood
predominate. At the end of forty-eight hours the active trypanosomes
are usually fewer in number and difficult to find. The surviving forms
do not differ much from those seen in the twenty-four hour old cultures
(fig. 35). During these two days, and also during the whole period
of cultivation some of the trypanosomes may show numbers of large
purple staining granules scattered through the protoplasm, the exact
significance of which at present seems to be uncertain (figs. 26,
49, and 52). No appreciable change in the position of the
blepharoplast is to be noted. In some cultures on the third, fourth,
and fifth days the trypanosomes may be so scanty as to have
apparently disappeared entirely from the medium, but usually they
may be found in these days although they may not be in great
numbers. Irregular clumps of trypanosomes formed around masses
of granular débris may be seen which are probably due to
agglomeration.

It is usually about this time in cultures, as well as in sub-cultures,
that large plasmodial masses with many nuclei and flagella
corresponding to 'some of the more exaggerated types of
degeneration forms' described by Bruce, etc. (1911b) in the gut of
the tsetse-fly appear (fig. 25). That some of these are degeneration
forms seems probable, because they are usually more frequently found
in cultures which die off quickly, and because they stain badly. But
all these multinucleated 'mis-shaped masses of protoplasm' do not
appear to be degenerated because some of them stain well, while
in fresh preparations some of them have been seen to give off by

a process of multiple fission daughter trypanosomes which are apparently normal (fig. 24).

It is at this period, third, fourth, and fifth days, that any distinct increase in size of the trypanosomes can be made out (figs. 4 and 10). Forms 30 μ in length by 3 or 4 μ in breadth are fairly common. The nucleus in these trypanosomes is large, oval in shape, compact, and well stained. The blepharoplast in some forms is rod-shaped, and has moved distinctly nearer to the nucleus giving the trypanosome a 'snouty' appearance. The protoplasm tends to stain a very dark blue and sometimes may contain numbers of purple granules, the so-called 'volutin' granules. The flagellum shows practically no free portion, and is seen to arise in many cases from a small red staining area immediately anterior to the blepharoplast. It is closely applied to the body of the trypanosome so that the undulating membrane is very narrow. These are very similar to the so-called 'female forms' figured by Kleine and Taute (1911).

The sixth, seventh, and eighth days. During this period there is usually marked multiplication of the trypanosomes in the tubes. On the sixth day the trypanosomes may be still scarce in numbers, but in most of the culture tubes this time seems to be the beginning of a period of great activity. The trypanosomes are all very large forms more than 40 μ in length have been observed (fig. 4), many of which are undergoing unequal longitudinal division (fig. 22). It is during these days that division rosettes are first seen (fig. 50). In the early stages these are composed of only a few trypanosomes (Pl. XXI, fig. 1), but later on colonies, similar to those described by Smedley (1905) in cultures of *T. brucei*, containing as many as a hundred or more, may be seen (Pl. XXI, fig. 2). The trypanosomes which compose these rosettes are attached centrally by their posterior ends, their flagellar ends being directed towards the periphery. This arrangement of the rosettes in *T. gambiense* and *T. rhodesiense*, with the flagella directed towards the periphery, resembles that of *T. brucei* as found by Novy and MacNeal (1904) and Smedley (1905), and differs from that of *T. lewisi*, where the flagella are directed centrally, as has been pointed out by these observers. In fresh specimens the individual trypanosomes show very active movements of the flagellar ends, and their protoplasm contains numerous highly refractile granules

which were not always observed in stained preparations. The
movements are so active that it is difficult to watch division take
place, but in small rosettes actual increase in numbers can be observed.
Frequently in association with the larger rosettes are seen rounded
bodies varying in size and filled with refractile granules, but what part
these take in the development is at present uncertain. Besides these
rosettes numerous free forms occur either singly or in pairs attached
by their posterior extremities. The form which predominates at
this period is one which corresponds exactly to that which Bruce
(1911b) calls the 'healthy normal developing type in the intestine
of the fly' (figs. 5 to 10, 43 to 49, and 53 to 61). We cannot do
better than give his description of this type. 'This is a long
moderately broad form, the protoplasm staining well, without
granules or vacuoles, having an oval compact nucleus situated
in the centre of the body, a small round micronucleus lying at some
distance from the elongated snout-like posterior extremity, the
undulating membrane, narrow and simple, and the flagellum proceeding
little, if anything beyond the protoplasm of the cell. The flagellum
also appears very frequently to arise from a pink-coloured body
situated near the micronucleus, an appearance never seen in the
normal blood trypanosome. This seems to be the healthy normal
developing type in the intestine of the fly.' In cultures these forms
were found to measure between 25 μ and 35 μ in length, and about
2 μ to 3 μ broad. The shape of the posterior extremities of these
forms varies. In some the 'snout' is blunt (figs. 5 to 8, 47 and 59),
while in others it is sharper (figs. 9, 43 to 46). The position of
the blepharoplast in relation to the nucleus in both *T. gambiense* and
T. rhodesiense varies from forms where it lies in close proximity to
the nucleus, to forms in which it is situated midway between the
nucleus and the posterior extremity of the trypanosome. We have
never seen any true crithidial forms in cultures. Such trypanosomes
have a very rapid and characteristic movement like that described
by Roubaud (1909) in similar forms found in *Glossina palpalis*. The
trypanosomes move forward with the anterior end in advance and
this has a peculiar wriggling movement, while the posterior portion
behind the blepharoplast remains rigid. About this stage of
development very long thin forms (15 μ to 30 μ in length by
0·75 to 1·5 μ in breadth) may be seen splitting off in fresh preparations

from some of the larger trypanosomes. This mode of division closely resembles the formation of the 'spirillar' forms (figs. 39 to 41) described by Leishman and Statham (1905) in cultures of *Leishmania donovani*. This may be seen occurring at any stage of the culture, but appears to be more prevalent about the eighth or ninth day. The movement of these forms is much more active than that of the stouter forms.

On the ninth, tenth, and eleventh days large numbers of the 'normal developing' forms, many of which are undergoing division, are found, but during this period a number of other types are seen, some of which correspond to the 'small developmental forms' of Bruce and his colleagues (1911b), and others are somewhat similar to the so-called 'male' trypanosomes of Kleine and Taute (1911). Numerous other forms are also present. The 'small developmental forms' (figs. 15 to 17, 42 and 58) are very similar to the 'normal developing forms,' and differ only in the facts that they are more stumpy in character and only measure about 20 μ by 3 μ, but intermediate types between these two forms may be found (figs. 18 to 20). The slender forms found in cultures resemble to a certain extent only the so-called 'male' trypanosomes of Kleine and Taute in that they are long and very slender, do not stain very deeply (taking up a pinkish colour), and have the blepharoplast fairly close to the nucleus, but differ from them because in no case have we seen the crithidial types depicted by these observers. These cultural forms resemble more closely the slender forms described by Bruce and his colleagues (1911b), and in our cultures were comparatively rare (figs. 11 to 14, 39 to 41 and 46). After the twelfth day the cultures slowly die off, but a few motile trypanosomes may be seen as late as the twentieth day. During this period very large, swollen, multinucleated trypanosomes may be seen which are very similar to those which Bruce calls the 'degenerative forms of the normal reproductive type.' (fig. 24).

The degeneration and disappearance of the trypanosomes in culture tubes is probably due to the exhaustion of the food supply, as well as to the production of waste products, to changes in the haemoglobin, and to evaporation. This is analogous to the statement of Bruce and his colleagues (1911b) that 'when a fresh supply of blood is taken in by the fly, this type (healthy normal developing

type) can be imagined to multiply with extraordinary rapidity. When the blood supply runs low then this type can also be imagined as degenerating and disappearing just as rapidly.' The sequence of changes described above in the cultures has been found in all our successful experiments, but the rate of development seems to depend on various factors, such as the incubation temperature, composition of the medium, etc.

(*b*) *Further History in sub-cultures*. Sub-cultures made between the seventh and tenth days were found to be more successful than those made at other dates. The reason of this appears to be that about this time the normal developing forms are more numerous. In these sub-cultures during the first days few or no trypanosomes may be seen, but about the seventh and eighth days small symmetrical division rosettes begin to appear attached centrally by their posterior extremities with the flagellar ends directed outwards. During the next few days these masses may increase in size up to many hundreds, and the culture fluid at the same time shows numerous very actively motile trypanosomes.

The duration of life in these sub-cultures varies as in the case of the original cultures from 10 to 17 days. In the case of *T. rhodesiense* the second generation showed very active development, but although the trypanosomes could be found in the third generation for several days, it was impossible to be sure that any actual multiplication had taken place in this sub-culture.

With *T. gambiense* on the other hand, sub-cultures were much more successful, and active multiplication was seen in the fourth generation as late as the thirty-seventh day. This sub-culture is still under observation. In some of these later sub-cultures of *T. gambiense* very large tangled masses, comprising many thousands of struggling trypanosomes, were seen presenting a very wonderful appearance. Such an enormous proliferation occurring at this time would appear to indicate that the trypanosomes had become accustomed to their new environment.

In all stages of the cultures rounded bodies may be present. Some of these, from their marked vacuolated appearance in fresh specimens and from their poorly stained protoplasm and fragmented nucleus, are evidently degenerate forms (fig. 29), but there frequently occur other forms which, from their definite outline, well-stained

protoplasm, compact nucleus and distinct blepharoplast (figs. 30 and 62), we do not think to be degenerate, but rather to be of a similar nature to rounded bodies described by Fantham (1911), who showed that rounded bodies could be inoculated into rats and produce trypanosomiasis. The same observer also showed that rounded or non-flagellated bodies grew and flagellated, and so turned into trypanosomes when placed in fresh, warm uninfected blood. Novy and MacNeal (1904) found similar rounded bodies in *T. brucei* cultures and concluded they were ' death changes,' because attempts to infect animals and obtain sub-cultures with them were negative.

On two separate occasions in sub-cultures of *T. rhodesiense* there was observed a definite cyst-like structure, 41 μ long by about 35 μ broad, with distinct capsule apparently composed of several layers, and this cyst was closely packed with hundreds of small oval bodies about 2 μ in length. Although kept under observation for over twenty-four hours no change in these bodies was observed. In spite of the absence of any evidence of bacterial or fungoid contamination we are unable to say whether this was a stage in life history of *T. rhodesiense* or merely a body of extraneous origin.

(*c*) *Comparison between the cultural character of T. gambiense and T. rhodesiense.* In our experience *T. gambiense* seems to be more easily cultivated than *T. rhodesiense*. Up to the present time sufficient cultures have not been carefully studied to state definitely whether it is possible to make a diagnosis between cultures of *T. rhodesiense* and *T. gambiense*. The life cycle and general morphology in both seems similar, but in all stages of the cultures of *T. rhodesiense* it is comparatively common to find forms both thin and stout with the nucleus in the posterior third or fourth of the trypanosomes (figs. 37 to 42, 47, 51, 58 and 60), that is posterior nuclear forms as described by Stephens and Fantham (1910). On the other hand in cultures of *T. gambiense*, although the nucleus may not be central, it is very rare to find forms with the nucleus even in the posterior third (fig. 21). This morphological difference has been well marked in the cultures examined during this investigation up to the present. On reference to the accompanying plates these differences are seen. These facts seem to strengthen and support the specific differences between *T. gambiense* and *T. rhodesiense*.

V. INFECTIVITY OF CULTURES

In cultures infectivity was retained as late as the third day, probably owing to the presence of some of the original blood forms inoculated, but after this day we have never succeeded in producing an infection though cultures of varying ages up to twenty-five days have been tried. As Bruce and his colleagues (1911a, 1911b) have found that the infectivity of *G. palpalis* seems to depend on the presence of trypanosomes of the blood type, which appear in the salivary glands about the twenty-fifth day, the non-infectivity of our cultures would seem to be due to the absence of these forms. It is possible that these forms appearing at a later date might render the cultures infective, but up to the present they have never been observed to occur in the cultures.

In the case of *T. lewisi*, Minchin and Thomson (1911) have found that the infectivity of the intestinal contents of an infected flea (*Ceratophyllus fasciatus*) depends upon the presence of stumpy forms which appear about the fifth or sixth day. The life cycle of *T. lewisi* in this insect host appears to differ from that of *T. gambiense* in the fact that the whole cycle is completed in the intestine, whereas in *T. gambiense* the infective forms are only found in the salivary glands. This might possibly be the reason why cultures of *T. lewisi* are infective from a very early date, whereas in *T. gambiense* it is possible that the developing trypanosomes may require some change to an environment similar to that of the salivary glands of the tsetse-fly before they regain their infectivity. Roubaud (1909) found that developmental forms of *T. gambiense*, somewhat similar to those found in the gut of *G. palpalis*, occurred in the intestines of mosquitos and *Stomoxys*, but quickly died out. These flies probably acted like culture tubes, and although they contained the non-infective developmental forms, because these flies were not suitable hosts, the cycle could not be completed and, therefore, infective forms did not develop. It is possible that the initial infectivity of cultures might be prolonged for a longer period than those observed by us, because the nearer an artificial culture medium approaches the normal blood conditions the longer will the trypanosomes retain their original characters and so probably remain infective, but in media resembling the conditions in the fly's stomach a development similar to that

which takes place in the fly will occur earlier and so infectivity will be lost sooner.

VI. COMPARISON BETWEEN THE CULTURAL FORMS AND THE FORMS FOUND IN *GLOSSINA PALPALIS*

From the description given above of the morphology of the cultural forms it will be seen that there is a very close resemblance between the changes which take place in cultures and those which occur in the gut of *Glossina palpalis*. It is interesting to note that Gray and Tulloch (1906) also drew attention to this feature in the cultures of *T. gambiense* attempted by them. A comparison of the cultural characters of *T. lewisi* with its development in the gut of the flea (*Ceratophyllus fasciatus*), Minchin and Thomson (1911), and the resemblance between the cultural forms of *Leishmania donovani* and the forms found in the probable insect host (*Cimex rotundatus*) (Patton, 1907, 1908) seem to strengthen the argument that the development of these Protozoa in cultures is similar to their development in their various insect hosts.

In the case of *T. gambiense*, both in cultures and in the gut of *Glossina palpalis*, the degeneration of a large number of the trypanosomes during the first few days takes place, leaving only a few stumpy forms, which seem to be the starting point of the new types found later in both. The close resemblance between the forms found in both has already been pointed out above in the description of the cultural morphology of the trypanosomes. Bruce and his colleagues (1911a, 1911b) have found that the developing forms found in the gut of *Glossina palpalis* are non-infective, and that it is only when trypanosomes like the blood types appear in the salivary glands that infectivity is found. So far, like these observers, we have been unable to infect animals by the injection of cultures containing only these ' normal developing forms.'

VII. SOME OBSERVATIONS ON THE SO-CALLED MALE AND FEMALE FORMS

Neither in *T. gambiense* nor in *T. rhodesiense* at any period of cultivation have we observed anything which might definitely be termed a sexual phase. No life-cycle such as described by Schaudinn in *T. noctuae* has been seen, and we have not found conjugation

similar to that observed by Prowazek (1905) and Baldrey (1909) in *T. lewisi* in the mid-gut of the rat-louse (*Haematopinus spinulosus*).

The fact that there are two distinct forms of trypanosomes, namely thin forms and stout forms, has suggested to some observers that there is a sexual cycle, and these forms have been called by Kleine and Taute (1911) male and female forms. There seems, however, to be no real reason for calling these male and female forms until it is definitely proved that conjugation takes place between them. Minchin (1908) was unable to find any definite evidence of conjugation between the 'male' and 'female' forms in *T. grayi*. Bruce (1909b) and Roubaud (1909), although finding so-called sexual forms also observed all stages of transition between these two extremes, and think there is not sufficient evidence to identify these forms with any stage in a sexual cycle. Roubaud believes that this differentiation of forms depends rather on the age of the culture and consequently on the composition of the medium. Walker (1910) experimenting with cultures of *Trypanoplasma ranae* comes to a somewhat similar conclusion. The occurrence in our cultures of trypanosomes attached by their posterior extremities was such a common phenomenon at certain periods, that it was at first thought that this might possibly be conjugation, but as the forms thus attached to each other were not always thin and stout forms these are rather to be considered either as the result of simple division or agglomeration. Such forms although watched for many hours have never been seen to fuse or apparently to make any attempt to do so. Baldrey (1911) has found similar forms in the gut of flies (*Tabanus* and *Stomoxys*) fed on animals infected with *T. evansi*, and he believes them to be conjugation forms, and has described the formation of an ookinete.

The very numerous varieties of trypanosomes found in our cultures, showing all stages between thin and stout varieties, seems to make it probable that the thin forms are merely due to the act of division.

In both fresh and stained specimens it would be very easy to mistake division phenomena for that of conjugation. On several occasions a large stout form has been seen with an apparent sphere of attraction thrown out to meet a long thin form which seemed to have its posterior end embedded in the protoplasm in close proximity

to the nucleus. Although this was watched for many hours, however, the thin form did not seem to make any further progress, and thus it is difficult to say whether this was an act of conjugation or not. No stained specimens, however, were obtained of this latter condition.

VIII. SUMMARY AND CONCLUSIONS

(1) *T. gambiense* and *T. rhodesiense* are capable of being cultivated on artificial media. *T. gambiense* has been cultivated for a period of thirty-seven days, and during that time was carried through four generations by means of sub-cultures. *T. rhodesiense* has been more difficult to cultivate and has continued to develop for only twenty-one days. All the flagellates disappeared in the third sub-cultures.

(2) The life history of these trypanosomes in culture tubes is similar to that which occurs in the gut of the insect host. This is shown by a comparison of the morphological characters of *T. gambiense* in cultures with those forms found by Sir David Bruce and his colleagues in *Glossina palpalis*. We find that the developmental forms found in the culture tubes are identical in their chief characteristics with those described by Bruce in the gut of an infected tsetse-fly.

(3) The cultures of *T. gambiense* and *T. rhodesiense* quickly lose their infectivity, and intraperitoneal injections of these into rats after the third day have been unsuccessful in causing an infection. This we have shown to be comparable to the results of Sir David Bruce and his colleagues, who have found that the intestinal contents of *G. palpalis* quickly become non-infective. The infectivity during the first few days appears to be due to the persistence of some of the original blood forms. In successful cultures, therefore, we expect infectivity to cease as soon as these blood forms disappear and the ' healthy developing forms ' which have been shown by Bruce to be non-infective appear.

(4) Our cultures of *T. gambiense* and *T. rhodesiense* have remained non-infective after the third day, and we believe that this is explained by the fact that the infective forms such as found by Bruce and his colleagues in the salivary glands of *Glossina palpalis* did not occur in our cultures, and we have suggested that probably

some tranference of the cultures to a new medium or environment similar to that of the salivary glands of the tsetse-fly might be required to permit the full life history of the trypanosomes being completed.

(5) Cultures of *T. rhodesiense* seem to differ from those of *T. gambiense*. In cultures of the former typical posterior nuclear forms such as described by Stephens and Fantham (1910) are relatively common, whereas in the latter they are of very rare occurrence.

(6) Although the so-called 'male' and 'female' forms are present in cultures we were unable to find any definite evidence of a sexual cycle.

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EXPLANATION OF PLATES

Trypanosoma gambiense

Figures drawn by means of an Abbé camera lucida, using Leitz ocular 4 and objective 1/12 inch. Stained Giemsa's stain. Magnification 1650.

PLATE XIX

Fig. 1. Slender trypanosome from blood of rat.

Fig. 2. Stout trypanosome from blood of rat.

Fig. 3. Stout form surviving after 24 hours in culture.

Fig. 4. Very large form with nucleus showing a karyosome (4th day of culture).

Figs. 5-10. Forms corresponding to the 'normal developing forms' described by Bruce in the gut of *Glossina palpalis*, and probably similar to the so-called 'female' forms of some observers. Note the long 'snout,' the position of the blepharoplast, the origin of the flagellum from a pink staining area anterior to the blepharoplast and the short or absent free flagellum. (From 10th, 11th, 18th, 17th, 29th and 5th days of growth). Nos. 7 and 8 from the second and No. 9 from the third generation. Fig. 10, dividing form.

Fig. 11. Form intermediate between the stout 'normal developing forms' ('female') and the slender forms ('male') (18th day of growth; second generation).

Figs. 12-14. Slender forms corresponding to the so-called 'male' forms. Note the long 'snouts' and homogeneous nuclei (11th, 10th and 12th days of growth).

Figs. 15-17. 'Small developing forms.' Forms shorter than the 'normal developing forms.' Note the varying position of the blepharoplast (14th, 14th and 15th days of growth, second generation).

Figs. 18-20. Forms intermediate between the 'normal developing forms' and the 'small developing forms' (15th, 18th and 31st days of growth, first, second and third generations respectively).

Fig. 21. Stumpy form with posterior nucleus. One of the very few posterior nuclear forms found in cultures of *T. gambiense* (15th day; second generation).

Figs. 22-23. Dividing forms (7th and 15th days).

Fig. 25. Large multinucleated plasmodial mass (4th day).

Figs. 24 and 26. Forms similar to those which Bruce calls 'degeneration forms of the normal reproductive type' (16th and 6th days).

Figs. 27-28.—Some forms seen when the cultures begin to die out (11th and 12th days).

Fig. 29. Rounded body with detached flagellum and fragmented nucleus (5th day).

Fig. 30. Rounded body with distinct nucleus and blepharoplast (12th day).

PLATE XIX

J. A. Sinton, del.

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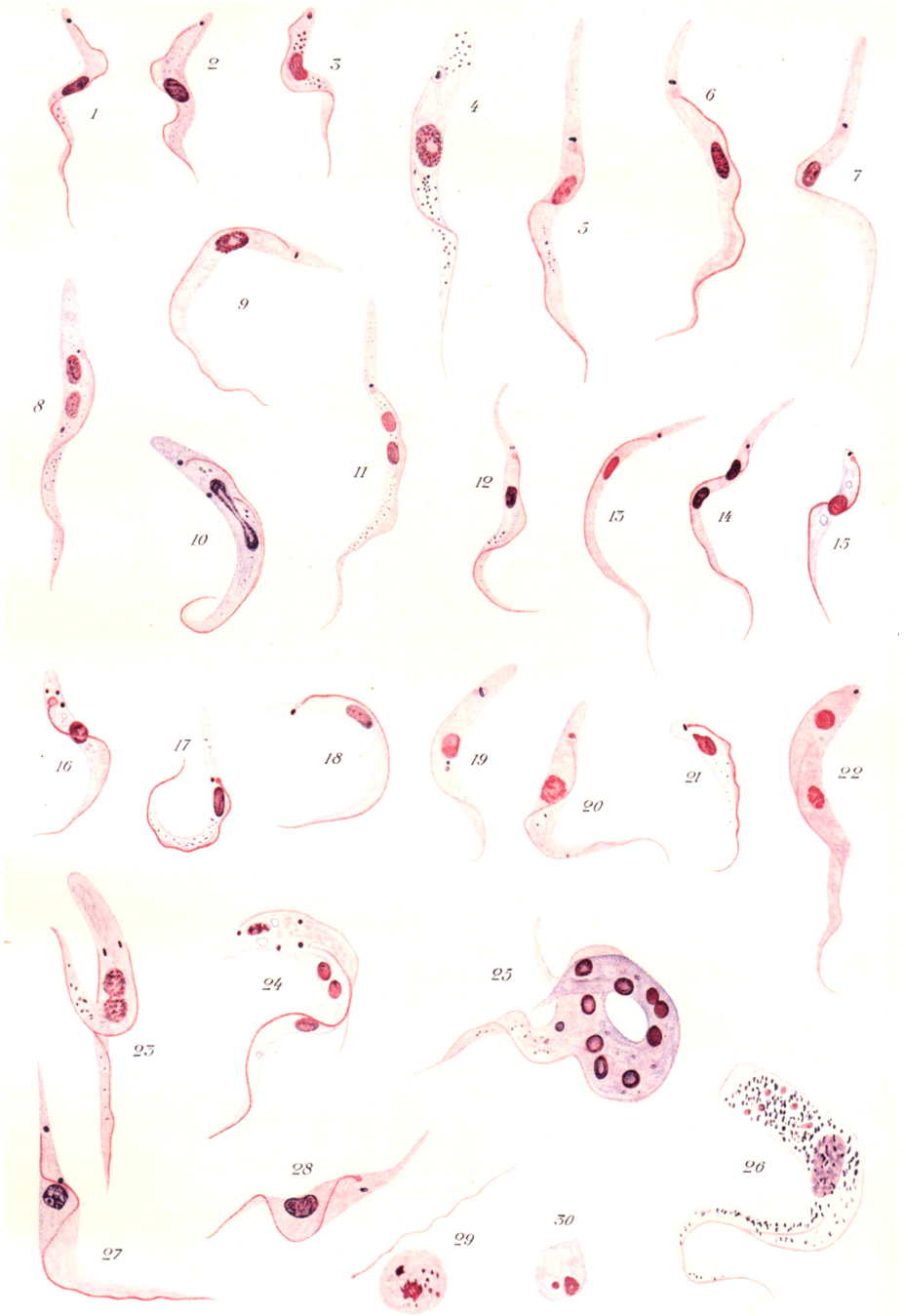
TRYPANOSOMA GAMBIENSE
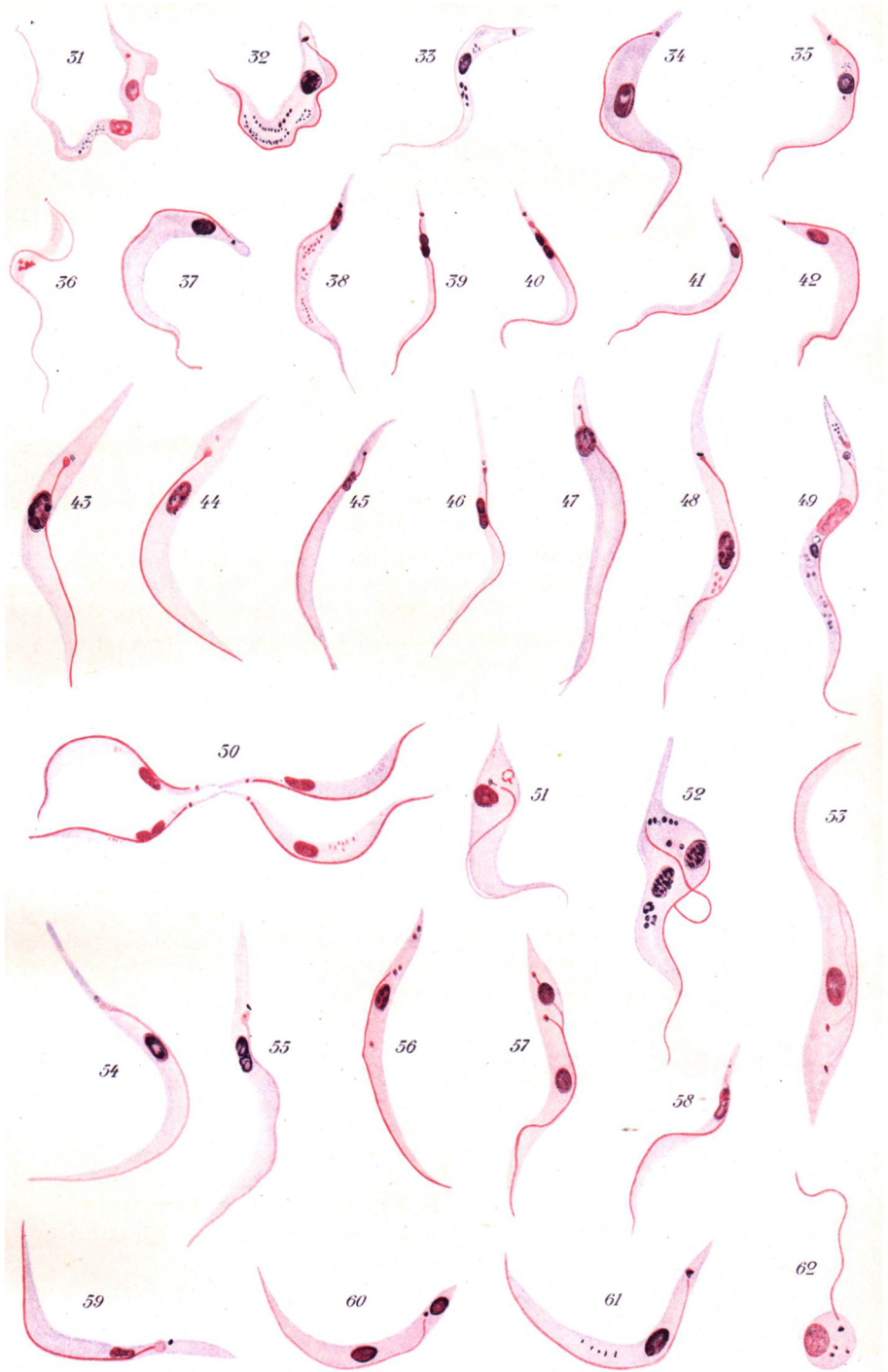IN CULTURES

PLATE XX

Trypanosoma rhodesiense

Slides stained with Giemsa. Figures drawn with Abbé camera lucida. No. 4 ocular and 1/12 inch oil immersion lens (Leitz). Magnification 1650.

Fig. 31. Normal blood form from peripheral circulation of a rat. Shows two nuclei and two blepharoplasts and well marked undulating membrane with free flagellum.

Fig. 32. Stout form from the peripheral blood of a rat showing the nucleus in a posterior position.

Fig. 33. Blood form after 24 hours in culture tube. Well stained protoplasm and nucleus. Rod-shaped blepharoplast and free flagellum.

Fig. 34. A stout form with sharp nose from a culture 24 hours old. Protoplasm stained a deep blue. No free flagellum.

Fig. 35. A short stout form from a culture 48 hours old, showing red stained area immediately anterior to the blepharoplast in which flagellum arises. Very short free flagellum.

Fig. 36. A degenerate form of long thin blood type after 24 hours in culture tube.

Fig. 37. Stout form. Posterior nucleus. Blunt aflagellar end. Very short free flagellum (third day).

Fig. 38. Form with posterior nucleus which is quite close to the blepharoplast. Short free flagellum. Flagellum closely applied to the body of the trypanosome (5th day of culture).

Figs. 39, 40, 41. Thin 'spirillar' forms, which correspond to the so-called 'male' forms. All show the nucleus in a more or less posterior position. There is no undulating membrane, and a very short free flagellum. (7th, 8th and 9th days of culture).

Fig. 42. Small developing form (8th day of culture). Posterior nucleus. No free flagellum. Flagellum closely applied to the body of the trypanosome.

Figs. 43 and 44. Normal developing forms (6th and 7th day of culture) with large oval nucleus, long 'snout,' and the blepharoplast moved away from the posterior end. These correspond to the so-called 'female' forms. They show also the red stained area immediately anterior to the blepharoplast. No free flagellum.

Fig. 45. Slender developing form, 8th day of culture. Showing nucleus in a posterior position.

PLATE XX

J. G. Thomson, del.

Firenze Lit A Ruffoni, Piazza S. Croce 20

TRYPANOSOMA RHODESIENSE
IN CULTURES

Fig. 46. Long thin form with very long 'snout.' No free flagellum.

Fig. 47. Normal developing form from the 9th day of a sub-culture with well marked posterior nucleus.

Fig. 48. Long form with oval nucleus and long 'snout' from 8th day of culture.

Fig. 49. Form showing presence of so-called 'volutin' granules from the 8th day of culture.

Fig. 50. Young division rosette 7th day of culture.

Fig. 51. Stout form from 8th day of culture with posterior nucleus.

Fig. 52. Large form showing division from 9th day of culture. Contains 'volutin' granules.

Fig. 53. Very large form with two blepharoplasts and a single large oval nucleus from 11th day of culture.

Fig. 54. Form from 8th day of sub-culture. Blepharoplast moved away from the posterior end, giving the appearance of a very long 'snout.' No free flagellum. The flagellum arises in red stained area immediately anterior to the blepharoplast.

Figs. 55-56. 'Normal developing forms' (12th and 15th days).

Fig. 57. Similar form with two nuclei and two blepharoplasts (12th day of culture).

Fig. 58. Posterior nuclear form. Small developmental form just after breaking away from a division rosette (see fig. 50). From 8th day of culture.

Fig. 59. Shows very broad 'snout' (from 10th day of culture).

Fig. 60. Form with two nuclei. One nucleus posterior (8th day of culture).

Fig. 61. Normal developing form (9th day of sub-culture).

Fig. 62. Well stained rounded body from a 3rd day's culture. Flagellum still attached.

PLATE XXI

T. rhodesiense in culture

Micro-photographs taken from slides stained with Giemsa.

Fig. 1. Division rosette from the 7th day of a culture. Shows the trypanosomes attached by their posterior ends. This arrangement in the rosettes is similar to that found in cultures of *T. brucei*.

Fig. 2. A colony of trypanosomes from a culture of *T. rhodesiense*. Slide made on the 8th day. Flagella are well stained, and also the nuclei. Note that the flagella are directed towards the periphery.
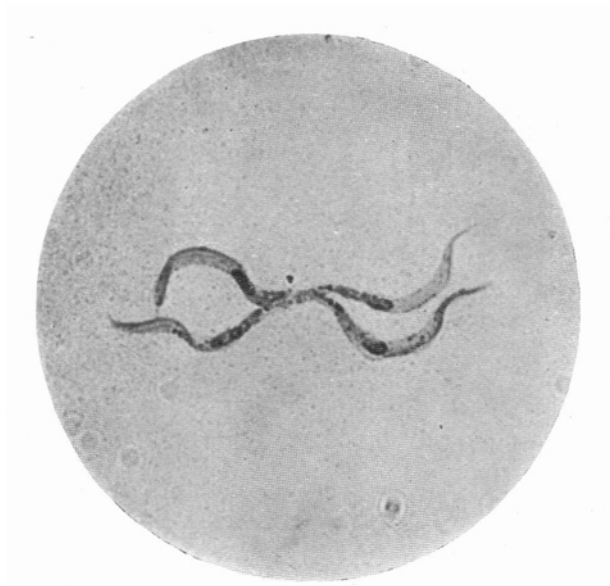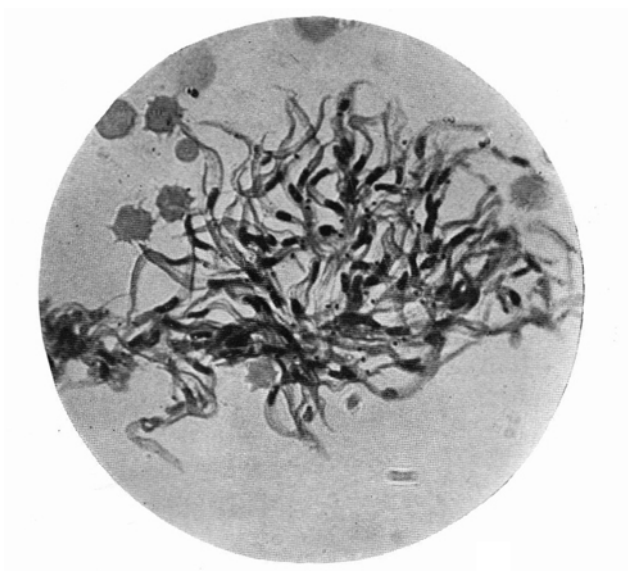
PLATE XXI.

Fɪɢ. 1.

Fɪɢ. 2.

Trypanosoma rhodesiense.

A. Lockes, photo.]

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