THE CULTIVATION OF TRYPANOSOMATA.

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By their researches on *Trypanosoma Lewisi* McNeal and Novy (VI. '03) have shown that cultures and subcultures of this protozoan parasite can be made with almost the same ease and certainty as cultures of bacteria. Hitherto the cultivation of protozoa has been successful to a very limited degree. Kartulis (1891) claimed to have cultivated *Amoeba coli* in an infusion of straw. He found however that multiplication did not occur, unless bacteria were present. Schardinger (1896) cultivated amoebae, in the presence of bacteria, on agar to which straw or hay infusion had been added. Tsujitani (1898), using Schardinger's method, cultivated "straw amoebae" in the presence of various bacteria, e.g. *S. cholerae*, *B. typhi*. By the application of heat, the bacteria were killed; but, in the presence of the nutriment afforded by the dead micro-organisms, the amoebae continued to multiply. All attempts to cultivate the amoebae in the absence of bacteria failed. The failure of many investigators to cultivate *Tr. Lewisi*, and other members of this group of haematozoa, did not appear remarkable, in view of the difficulty of producing artificially the conditions under which these parasites exist in the blood of their hosts. A year ago Novy and McNeal announced that they had succeeded in cultivating *Tr. Brucei* and a few months later they successfully cultivated the *Surra* Trypanosome of the Philippines (see page 44). It therefore seems probable that their methods will be found applicable for the cultivation of other species of trypanosomes.

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Various forms of Trypanosomiasis.

Recent discoveries have added considerably to the number of species included in the genus *Trypanosoma*. In the accompanying table (p. 26) is given a list of the chief forms of Trypanosomiasis, which are at present considered to be due to infection with distinct species of Trypanosoma. No order of the Vertebrata is exempt from this form of parasitism, and it is also probable that certain species of Trypanosomes produce morbid conditions in the blood-sucking insects, through whose agency these diseases are usually propagated. It is generally accepted that these insects act merely as "carriers" of the disease, since there is no evidence of their acting as intermediate hosts[1].

Differentiation of the various forms of Trypanosomiasis.

Until recently it was doubtful whether some of the varieties of Trypanosomiasis, mentioned in the accompanying table (p. 26), were caused by distinct species of Trypanosomes. For instance it was found to be impossible to distinguish with certainty *Nagana* from *Surra*. Minute differences in the morphology of the two parasites causing these diseases have been described, but differences almost as great exist between individual trypanosomes of the same species. The clinical symptoms are identical in the two diseases and the pathological changes, which are very few, are not distinctive. Infection experiments on animals have not proved conclusive and have caused some confusion owing to the variations in susceptibility of the same species of animal in different parts of the world. For the same reasons *Mal de Caderas* has been confused with *Dourine* and *Surra*. Immunising experiments, notably those of Nocard and of Laveran and Mesnil, have recently proved to be of great service in distinguishing these diseases. Nocard (4. v. '01, p. 466) produced a fatal infection with *Nagana* in two dogs, which had been previously immunised to *Dourine*. Laveran and Mesnil (22. vi. '03, p. 1529) have immunised a goat successively to *Nagana*, *Mal de Caderas* and *Surra*. Their experiments extended over a period of nearly two years. They reported subsequently (1904, p. 134) that this goat succumbed to an infection with the Horse Trypanosome of Gambia

[1] Schaudinn (1904, p. 387) states that *Halteridium* in the owl (*Athene noctuae*) is the sexual stage of a Trypanosome (*Tr. noctuae*, Celli and Sanfelice), which undergoes a complex form of multiplication both in the gnat (*Culex pipiens*) and in the blood of the owl, and then gives rise to the sexual forms of *Halteridium*.

Trypanosomata and their Hosts, &c.

Species of parasite	Discovered by	Host	Common name of the disease	Propagation of the disease by	Geographical distribution of the disease	Pathogenicity
Tr. of the Trout	Valentine (1841)	Salmo fario	——	——	——	——
Tr. rotatorium (Mayer)	Mayer, Gruby (1843)	Frog	——	——	World-wide	No evidence of pathogenicity for frogs or other animals.
Tr. Lewisi (Kent 1880)	Lewis (1878)	Rat	——	Fleas, ? Lice	World-wide	Only causes death of rats occasionally. A transient infection is caused in guinea-pigs.
Tr. Evansi (Steel 1885)	Evans (1880)	Horse &c.	Surra	? Tabanus tropicus, ? Stomoxys calcitrans	India, Burma, Philippine Islands, Mauritius	All domesticated animals, rats, monkeys and most other wild animals.
Tr. cobitis	Mitrophanov (1883)	Mudfish (*Cobitis fossilis*)	——	——	——	——
Tr. avium	Danilewsky (1885)	Birds	——	——	——	——
Tr. Brucei (Plimmer and Bradford 1899)	Bruce (1895)	Horse &c.	Nagana or Tsetse-fly disease	Glossina morsitans	S. Africa, W. Africa	Similar to Surra.
Tr. equiperdum (Doflein 1901)	Rouget (1896)	Horse	Dourine, Mal du coit	coitus	S. Europe, N. Africa	Similar to Surra.
Tr. —	Nepveu (1898)	Man	?	——	Algiers	——
Tr. equinum (Vosges 1901)	Elmassian (1901)	Horse	Mal de Caderas	? Tabanus, ? Stomoxys calcitrans	S. America	Similar to Surra.
Tr. gambiense (Dutton 1902)	Forde & Dutton (1901)	Man	Trypanosome fever. Now regarded as an early stage of Human Trypanosomiasis	? Glossina palpalis, ? Tabanus dorsovitta	Gambia	Monkeys, guinea-pigs, rabbits, rats, dogs, sheep, goats, etc.
Tr. ugandense (Castellani 1903) Tr. Castellani (Kruse 1903)	Castellani (1902)	Man (Uganda)	Sleeping-sickness. Now regarded as the late stage of Human Trypanosomiasis	Glossina palpalis	Uganda, Congo Free State	Similar to Tr. gambiense.
Tr. Theileri (Laveran, Bruce, 1902)	Theiler (1902)	Cattle	Gall-sickness	Hippobosca rufipes	S. Africa	Cattle only.
Tr. transvaaliense (Laveran 1903)	Theiler (1902)	Cattle	Similar to, if not identical with, Gall-sickness	——	Transvaal	Similar to Gall-sickness.
Tr. dimorphon (Dutton and Todd)	Dutton and Todd (1903)	Horse	——	? Glossina palpalis	Gambia	Similar to Surra.

(*Tr. dimorphon*). Similarly Lignières (1902, p. 112) has produced a fatal infection with *Mal de Caderas* in two dogs, which had been immunised to *Dourine*, and Nocard, Vallée and Carré (19. x. '03, p. 624) have infected a cow, which was immune to *Nagana*, with *Surra*. The cultural methods, introduced by McNeal and Novy, are likely to prove of great value in distinguishing one species of Trypanosome from another. These workers have already been able to thus confirm the results of the immunising experiments (mentioned above) which indicated that *Surra* and *Nagana* were distinct diseases (see p. 44).

Classification of the genus Trypanosoma.

Salmon and Stiles (1902, p. 29) give the following classification :—

Class :— *Mastigophora.*
Subclass :—*Flagellata.*
Order :— *Monadida*— Independently living organisms, but in some cases they form colonies. The number and arrangement of the flagella varies. There is sometimes an undulating membrane.
Family :— *Trypanosomidae*—Includes the genus *Trypanoplasma*, parasitic organisms resembling the *Trypanosoma* but possessing two flagella, one of which is situated at each end.
Genus :— *Trypanosoma.*
Species :— *Tr. rotatorium* (Mayer), *Tr. Lewisi*, etc.[1]

Morphology of Tr. Lewisi.

It is unnecessary to describe in detail the morphology of the adult form of this well known parasite, but on page 37 will be found a summary of the chief differences between the parasitic and cultural forms of *Tr. Lewisi*. It will be convenient, however, to consider at this point the modes of multiplication of these parasites, and to describe the morphology of the young forms, which closely resemble the cultural forms of *Tr. Lewisi*. The following account is taken from the description given by Laveran and Mesnil (1901, p. 684), who have extended the researches of Rabinowitsch and Kempner (1899) and of Wasielewski and Senn (1900). Two modes of multiplication of *Tr. Lewisi* are described.

[1] Laveran and Mesnil (1904, p. 41), in describing the cultural forms of *Tr. Lewisi*, remark on the resemblance of these organisms to the species included under the genera *Herpetomonas* (Kent) and *Crithidia* (Léger). [Fusiform parasites, possessing long flagella but *no* undulating membrane. Their centrosomes are placed *anteriorly* to the nucleus.] They consider therefore that the genus *Trypanosoma* should be included in the family *Cercomonadidae*, to which *Herpetomonas* belongs.

(1) *Longitudinal division.* The trypanosome increases in length, measures 35μ or more, and becomes three or four times as broad as usual. The nucleus and the centrosome enlarge, the latter elongating, and the two structures are approximated. Simple division (amitosis) of the nucleus occurs. The base of the flagellum thickens and divides at the same time as division of the centrosome occurs, which process may follow or precede division of the nucleus. By the splitting of the flagellum along part of its length a new flagellum is formed, which elongates rapidly. Subsequently the protoplasm divides. The two organisms, that are thus formed, are frequently very unequal in size. It is customary therefore to speak of mother cells and daughter cells. The latter may only measure a few microns in length.

(2) *Multiplication by segmentation.* The trypanosome becomes spherical, ovoid or irregularly shaped. The nucleus and centrosome divide and subdivide a variable number of times, new flagella are formed and the protoplasm indents and divides. In this way the rosettes, which are found in the blood during the early stages of infection, are formed. In Fig. 3, Plate IV. is shown a rosette forming by the process of segmentation. By further division a rosette, consisting of numerous young forms, which are arranged radially with their flagella directed outwards, is formed (see Fig. 4, Plate IV.). The young form differs from the adult form of *Tr. Lewisi* in the following respects: (1) Its body is fusiform in shape and much smaller. (2) The nucleus is more centrally placed. (3) The centrosome is placed close to, and not infrequently anteriorly to, the nucleus. (4) The undulating membrane is not developed or is too small to be distinguished.

I. *Cultivation of Tr. Lewisi.*

(1) *Preparation of media.* The medium, employed by McNeal and Novy, consisted of a mixture of agar and defibrinated blood. Neutral or slightly acid agar was used. The following method of preparing this medium is essentially the same as that described by McNeal and Novy. (*a*) Agar: To nutrient broth, prepared from bullock's heart without the addition of alkali and containing 1% of peptone and salt, is added 2% of agar. The agar is then cleared in the usual way, by the addition of white of egg, then placed in test-tubes and sterilized. (*b*) Defibrinated blood: The blood is collected from the heart of a rabbit, by means of sterilized Pasteur bulbs, and is then defibrinated in sterilized bottles, containing a few fragments of broken glass. (*c*) Rabbit-blood-agar:

To test-tubes, containing about 5 c.c. of melted agar, which has been cooled to about 45° C., is added an equal volume of defibrinated blood. After intimately mixing the blood and agar, the tube is slanted and the medium sets in a firm slope. Soon after solidification, the tubes must be placed in a nearly vertical position, so as to obtain a large amount of fluid of expression.

Precautions to be taken in preparing medium :—

(1) Strict asepsis must be ensured.

(2) Agar must be well cooled, otherwise the red blood corpuscles are broken up and a brown medium, containing haematin, is obtained.

(3) Fresh blood only should be used.

(2) *Inoculation of medium.* Two or three loopfuls of blood, taken from the heart of an infected rat, are added to the fluid of expression in the tubes of blood-agar. A larger quantity should be added, by means of a pipette, if the animal is not suffering from a severe infection. Subcultures are made by transferring one or two loopfuls of the fluid of the culture to a fresh tube of blood-agar.

(3) *Incubation of cultures.* McNeal and Novy found that *Tr. Lewisi* was best cultivated at a low temperature. Incubated at 37° C., cultures grew quickly but rapidly degenerated owing to the alteration of the blood-medium. Cultures, placed in the cool incubator (20—25°) or kept in the dark at room temperature (18—20° C.), contained numerous colonies at the end of three weeks' time and remained alive for several months. They state that evaporation should be prevented by sealing the tubes with rubber caps.

Viability of Tr. Lewisi in cultures.

It is apparently possible to cultivate *Tr. Lewisi* indefinitely; thus McNeal and Novy, in their first report, stated that they found no signs of degeneration in these organisms after cultivating them for nearly a year through eleven generations. They have announced since then (v. '04, p. 3) that this culture has been cultivated for twenty-six generations during a period of two years. The culture, which I made nine months ago, has been subcultivated through as many generations with similar results. The last generation multiplied rapidly and was quite as infective as the preceding ones.

Culture 1. *Incubated at* 20° C. The first culture was made by me on March 18, 1904. One drop of blood, taken from the heart of a white rat, was added to a tube of rabbit-blood-agar. The white

rat had been injected intra-peritoneally fourteen days previously with blood taken from an infected sewer rat[1]. Trypanosomes were found in the blood of the white rat on the fourth day after inoculation, and, at the time of its death, its blood was swarming with parasites. A drop of the culture, examined a few hours after it had been made, contained about six, actively motile, trypanosomes per field. On the fourth day the trypanosomes were not so numerous, some of them were pale and motionless, but the majority of them were very active. On the fifth day, a few small "agglomeration rosettes," composed of four to six trypanosomes, were found. The trypanosomes, which were loosely linked together at their posterior extremities, were arranged in a symmetrical manner. Their long flagella could be seen actively lashing at the periphery of the rosette. On the tenth day, small *colonies*, containing twenty or more trypanosomes, were numerous. Most of the organisms appeared to be attached to the centre of the colonies by their flagellar ends. The structure of the colonies will be described subsequently on p. 32. Free forms were numerous and exceedingly active. Pairs of trypanosomes, which were attached to one another by their posterior extremities, were common. On the twenty-first day the culture was swarming with colonies and free forms. At the end of eight weeks the culture contained numerous colonies of large size and many, actively motile, free forms; but a month later the trypanosomes were very granular in appearance and the majority of them were motionless.

Some sterile salt solution was added to this culture on two occasions to counteract evaporation, but no fresh blood was added in order to prolong the life of the culture.

Generation 2. Three loopfuls of culture 1, the latter being six weeks old, were added to a tube of rabbit-blood-agar. On the fourth day a drop of the culture contained several large masses of trypanosomes evidently formed by the partial disintegration of the colonies. Many of the trypanosomes were motionless. Active free forms were not numerous. On the fifteenth day there were numerous colonies, some being of large size. On the twenty-first day the culture was swarming with colonies and free forms. The remaining subcultures behaved similarly and need not be further described.

[1] Five out of thirteen rats (38·4 %), caught in a certain locality near Cambridge, were found to be infected with *Tr. Lewisi*.

Generation 2 made from generation 1 on forty-second day.
„ 3 „ „ „ 2 on fifteenth day.
„ 4 „ „ „ 3 on twenty-first day.
„ 5 „ „ „ 4 on twenty-third day.
„ 6 „ „ „ 5 on ninth day.
„ 7 „ „ „ 6 on thirty-first day.

Another culture of *Tr. Lewisi* was cultivated through a like number of generations, and numerous parallel subcultures were made either from this series or the one mentioned above.

Infection experiments on rats with cultures of Tr. Lewisi.

Rat 1. A white rat received intra-peritoneally three loopfuls of a culture of the third generation which was seventeen days old. Parasites were found in the rat's blood on the fifth, and were numerous on the tenth day.

Rat 2. A white rat was injected intra-peritoneally with six drops of a culture of the fourth generation which was thirty-one days old. Parasites were found in the blood of the rat on the second day. "Multiplication rosettes" and young forms were numerous on the fourth day. A few days later the blood was swarming with parasites.

Infection of rats with cultures belonging to another series.

Three loopfuls were injected intra-peritoneally in each case.

Rat A. A white rat was injected with a culture, which was thirty-three days old: culture belonged to the third generation of the series. Parasites appeared in the blood on the sixth day.

Rat B. A culture of fourth generation, which was thirteen days old, was used. Parasites appeared in the blood on the sixth day.

Morphology of the rat trypanosome in cultures.

(A) *Unstained preparations.* The parasites found in cultures of *Tr. Lewisi* vary greatly in size, shape and motility. The variability is especially marked in young cultures containing a large number of active free forms. In a hanging-drop, made from such a culture, the following forms may be recognised:

(1) *Exceedingly motile, slender forms.* The body of the trypanosome is bright and glistening and apparently homogeneous in structure, posteriorly it ends in a sharp pointed extremity, whilst anteriorly it is

prolonged into a stiff flagellum, which is sometimes twice the length of the body of the organism. No undulating membrane is visible and the movements of the organism appear to be due entirely to the rapid "vibratory" action of the flagellum. The excursion of the flagellum is extremely limited, but this is compensated for by the rapidity with which the side-to-side movements are made. Usually the trypanosome darts across the field in a straight line, but occasionally it moves in a circular direction. After describing the greater part of a circle, often more than once, it may dart off at a tangent. During its motion, a rapid rotatory movement of the whole parasite, on its long axis, may often be observed.

(2) *Shorter, spindle-shaped forms.* Trypanosomes similar to the first form.

(3) *Large pear-shaped forms.* The posterior extremity is broad and rounded. The body of the trypanosome tapers suddenly and ends in a long flagellum, which is thrown into a series of folds by the passage of wave-like contractions. This is probably an involution form, its movements are, as a rule, sluggish.

(4) *Small pear-shaped forms.* These are young, very active forms. The body of the trypanosome is small, rounded at one end, and tapers at the other end into a long flagellum.

(5) *Involution forms.* Spherical forms, pale or granular in appearance, are common in older cultures. They possess one or more active thin flagella, but do not usually exhibit more than a slight swaying movement.

(6) *Colonies.* All the forms, described above, may be found in the colonies of trypanosomes. The colonies increase rapidly in size, at first they consist of a few trypanosomes, grouped loosely together in the form of a "rosette." The trypanosomes are attached to one another at their anterior extremities by their flagella, which converge to the centre of the colony, and each individual has independent and very active movements, the body of the trypanosome being waved rapidly, from side to side or to and fro, by the action of its flagellum. The colony, if seen with its individuals all lying in the same plane, presents a beautifully symmetrical appearance. The smaller colonies move actively about, but the larger only possess a slight swaying motion. Colonies, composed of many hundreds of trypanosomes, are found in cultures which are two or three weeks old. The trypanosomes are grouped together in compact, almost spherical masses, which have the same symmetrical appearance as the smaller colonies. The older cells

in the central portion of the colony are generally spherical in shape and granular in appearance; at the periphery the cells are spindle-shaped and exhibit very active movements. In the central portion of the colony tangled masses of flagella, actively contracting, may sometimes be seen. In three or four weeks' time the colonies grow to an enormous size and contain many thousands of trypanosomes. These masses of trypanosomes are visible to the naked eye; they form small soft granules of a yellowish white colour. It is possible that these larger colonies are formed by the clumping together of several smaller colonies, but in the latter several centres of growth may often be observed. This arrangement of the colonies is shown clearly in Fig. 2, Plate V., which is a photograph of part of a large colony. At least five distinct, smaller, colonies, arranged eccentrically, can be seen. Fig. 1, Plate V. is from a photograph of a young colony, whose organisms are radiating from two centres. It shows the shape and comparative freedom of the individuals in a young colony. Fig. 1, Plate IV. is from a drawing of a colony, which has two centres of development. The angularity and the compressed appearance of the cells suggest that this colony was separated off from a much larger mass of trypanosomes. This colony and the larger one, shown in Fig. 2, Plate V., were found in the same film. (The figures referred to are made from stained preparations.)

(B) *Stained preparations*[1]. Certain details of the structure of the

[1] *The Staining of Trypanosoma in Cultures.*

At first I experienced considerable difficulty in obtaining well stained preparations. Failures were due to several causes: (1) The serous fluid of the blood-agar contains in suspension degenerated blood elements and small lumps of agar, so that even thin films thereof make opaque preparations, which are useless for studying the morphology of the organisms. (2) Colonies of trypanosomes stain deeply and only a blurred outline of the whole mass is seen. (3) Romanowsky's stain, and similar stains, deposit masses of precipitate and fail to bring out any detail in the organisms. By the use of the "Borrel-blue" stain, introduced by Laveran and Mesnil for staining trypanosomes in blood-films, beautiful preparations may be obtained, which show clearly the structure and detail of the colonies.

"Borrel-blue" is made by adding a small quantity of freshly precipitated silver oxide to a saturated watery solution of Methylene-Blue (Höchst). The following directions for staining are given by Laveran and Mesnil ('01, p. 680). (1) Make a thin film and place it, as soon as it is dry, in absolute alcohol for 5 minutes. (2) Transfer the film, without drying, to the stain which is composed of:

Borrel-blue	1 part.
1 % Eosine (Höchst)	4 parts.
Distilled water	6 parts.

The film must be placed in the stain the moment the latter is mixed. (3) After staining

trypanosomes and colonies, which can only be seen in stained preparations, remain to be described.

(1) *Protoplasm.* The protoplasm of the cell is usually homogeneous in structure. It stains a pale blue, or, if the preparation has been stained for a long time, a pale violet. Small, deeply stained, granules of a red or violet colour are sometimes seen but they are seldom numerous. Vacuolisation of the protoplasm is rare, but occasionally a large highly refractile vacuole, whose diameter is nearly equal to the width of the trypanosome, is found. [These vacuoles have a greenish tint when seen in the living parasite.]

(2) *Nucleus.* The position of the nucleus is variable. In young pear-shaped forms it is usually placed at one side of the cell in the anterior half of the body of the trypanosome. In the longer spindle-shaped forms the nucleus is more centrally placed. The nucleus is round or oval in shape, and stains a light purple-red colour.

(3) *The Centrosome.* An intimate connection appears to exist between the nucleus and the centrosome. In young forms these structures are always found close to one another, in some cases the centrosome appears to be embedded in the peripheral portion of the nucleus, from which however it is easily distinguished by its staining deeply. The centrosome is usually placed either at one side of the nucleus or at a variable distance anteriorly to it. In free forms, at any rate, I have never observed it lying posteriorly to the nucleus. The centrosome is most commonly seen as an elongated, rod-like, structure, with sharply defined edges, lying transversely to the long axis of the trypanosome. Sometimes it has a crescentic outline and occasionally is represented by two small round bodies, placed in close apposition.

the film for 15 minutes, wash it well in water. (4) Place the film in 5% tannic acid solution for 10 minutes. (5) Wash the film again in water and dry it; if a precipitate is deposited on the film, clear in clove oil and then wash it in Xylol. (6) Mount the preparation in Canada Balsam.

On account of the dense precipitate which is deposited, I found it advisable to place the coverslips in the stain with their film-surface downwards. Any precipitate, adhering to a film, may be easily removed by immersing the coverslip in water and brushing it lightly with a camel's hair brush.

When this method of fixing and staining is used, the outlines of the organisms are well preserved. The protoplasm stains a pale blue, the nucleus a bright reddish-violet colour. The flagellum stains either a bright red or the same colour as the nucleus. By prolonged staining (12–24 hours), excellent preparations are obtained for studying the outline and structure of the Trypanosomes in colonies. The coloration is however somewhat different and is less intense (see Fig. 1, Plate IV. where the protoplasm of the cells has a pale violet tint).

The centrosome is apparently homogeneous in structure, it stains a deep violet or crimson colour.

(4) *Flagellum.* In well stained preparations nearly every trypanosome shows a flagellum, which arises from the centrosome. The flagellum takes a straight or slightly sinuous course and passes, apparently, through the protoplasm of the cell. The free portion of the flagellum is often two, three, or four times the length of the body of the trypanosome. The centrosome is sometimes situated so near to the anterior end of the body of the trypanosome that the flagellum is almost entirely extracellular. The flagellum stains a bright red colour.

(5) *Undulating membrane.* It is doubtful whether this structure exists in the cultural forms of *Tr. Lewisi.* If present the undulating membrane must be exceedingly small, for the flagellum projects from the body of the trypanosome as a free structure for almost its entire length.

(6) *Colonies.* There is little to add to the description, given above, of the structure of the trypanosomes and of their arrangement in the colonies. Fig. 2, Plate IV., from a drawing made of a stained preparation of a colony, which has been much flattened out, shows that the trypanosomes sometimes possess very long flagella. The bunching together of these structures in the centre of the colony is better shown in the other illustrations. McNeal and Novy, in their description of cultures of *Tr. Lewisi,* call these masses of trypanosomes "rosettes," but in their paper on the cultivation of *Tr. Brucei* they also use the term "colony." The latter term seems to me to be more suitable for the following reasons:—(1) Among the Protozoa colony-formation is not infrequent and results from the incomplete separation of daughter-cells from their mother-cells. (2) A second reason for using the word "colony" instead of "rosette" is that the latter term has been already applied to the groups of multiplication forms found in rats during the early stages of infection. In Fig. 4, Plate IV., is given a drawing of one of these rosettes for the purpose of comparison.

The trypanosomes contained in the rosette are similar in shape to many of the organisms found in colonies in cultures of *Tr. Lewisi,* but they are attached at their posterior extremities and their flagella radiate outwards.

Novy and McNeal (1. '04, p. 28) have not apparently succeeded in staining the flagella in their preparations, though they noted the position of the centrosome. They expressed the opinion that the end of the trypanosome, pointing towards the

periphery of the colony, was the anterior extremity and that from it a flagellum would arise if the cultural conditions were perfected.

Measurements of the cultural forms of Tr. Lewisi.

Pear-shaped forms:

(1) Body. 3·6—4·4μ long and nearly as broad.

(2) Flagellum. Two to four times the length of the body.

Spindle-shaped forms. 14—16 × 2·4—3·5μ (flagellum not included).

Smaller and larger forms are frequently found. Novy and McNeal (I. '04, p. 26) give measurements varying from 2—60μ. The adult, parasitic form of *Tr. Lewisi* measures 24—25 × 1·5μ (Laveran and Mesnil, 1901, p. 681).

Mode of multiplication in cultures of Tr. Lewisi.

The mode of multiplication of these parasites in the rat has been already described (p. 28). The trypanosomes appear to multiply in cultures in a similar manner. In a young culture, free forms, dividing longitudinally, are common, but, as a rule, the mother-cell cannot be distinguished by its greater size. In stained preparations, forms which are undergoing *segmentation* are occasionally seen; the cells contain numerous nuclei and centrosomes from which arise flagella, and the protoplasm is incompletely segmented. Some of the trypanosomes found in cultures are exceedingly minute, small enough to pass through a Berkefeld filter (Novy and McNeal, I. '04, p. 28).

Degeneration of the cultural forms of Tr. Lewisi.

The faint shadowy forms of degenerated Trypanosomes remain practically unstained, with the exception of their centrosomes and flagella. Usually the outline of the cell is indicated faintly by dots of stain of a pinkish colour. Flagella, which have become detached from their trypanosomes, are frequently seen in cultures. They are found lying either singly or in clusters. The centrosome is often found still to be attached to the flagellum. Similar objects have been described in the blood of infected rats by Laveran and Mesnil.

Summary of the chief differences in morphology between the cultural and the parasitic (adult) forms of Tr. Lewisi.

Cultural forms.	*Parasitic forms.*
Exceedingly active.	Very active.
Very variable in size and shape, generally spindle-shaped or pear-shaped.	Size only varies within narrow limits. Body is slightly fusiform and has sharp pointed extremities.
Nucleus is variable in position.	Nucleus is invariably situated at the middle of the anterior half of the body of the parasite.
Centrosome is found either close to the nucleus or at a variable distance anteriorly to it. It is usually elongated.	Centrosome is found at a short distance from the posterior extremity and is usually round.
Undulating membrane is not developed.	Undulating membrane is well developed and is usually thrown into one or two folds.
Flagellum is frequently very long. Its basal portion is very short owing to the position of the centrosome.	Flagellum is much shorter, relatively to the length of the body of the parasite. If traced backwards in the free border of the undulating membrane, the flagellum is seen to arise from the centrosome.

II. *Cultivation of Tr. Brucei.*

Tr. Brucei can be cultivated on blood-agar in the same way as *Tr. Lewisi* but with much greater difficulty. Novy and McNeal (2. I. '04, p. 6) using the blood of different animals, infected with Nagana, for inoculating the media, failed to secure cultures twenty-five times in succession. In another series of experiments they were successful in four out of twenty-five attempts at cultivation. Subcultivation proved to be a more certain and rapid process. In their preliminary notice Novy and McNeal stated that one of their cultures was in its eighth generation, the period of cultivation having extended over a hundred days. In a later paper (V. '04, p. 3) these authors state that one of their cultures, started on August 27th, 1903, is now in its twenty-seventh generation. No mention is made of the virulence of the culture.

There are differences between *Tr. Lewisi* and *Tr. Brucei* which may account for the difficulty in cultivating the latter. Nagana blood, kept *in vitro*, rapidly loses its infectivity. Kanthack, Durham and Blandford (X. '98, p. 117) state that the parasites sometimes become motionless within twenty minutes from the time of collecting the blood, but generally motile forms are found for two or three days and occasionally for five or six days. It was exceptional to find that the blood was

infective at the end of three or four days. The rat trypanosome, on the other hand, may be maintained alive for more than fifty days, as shown by Laveran and Mesnil (1901, p. 679), if the blood is collected aseptically and kept cool in an ice-chest. The rapid alteration of the Nagana trypanosomes on change of environment, probably accounts for the difficulty in obtaining cultures of these organisms. In blood-agar, the life of the Nagana trypanosome is greatly prolonged, although there may be no signs of multiplication of the parasite. I have found involution forms, possessing active flagella, three weeks after adding the infected blood to the medium.

It is well to note the most favourable conditions for cultivating *Tr. Brucei.* The quantity of blood added should be at least equal in volume to the agar which is used. On account of the slow rate of multiplication of the trypanosomes and the rapidity with which cultures degenerate, the latter are best incubated at a temperature of 25° C. (see incubation of cultures of *Tr. Lewisi,* p. 29).

Three out of ten attempts which I made to cultivate *Tr. Brucei*[1] were successful. Two of the cultures were obtained from white rats and the third culture was made from a rabbit. The unsuccessful attempts were made with rats (2), mice (2), rabbits (3). Rabbits are not suitable animals from which to obtain cultures because their blood contains few parasites. Mice are inconveniently small to work with. I think that if I had confined my cultural experiments to rats there would have been a smaller percentage of failures.

Generation 1. Incubated at 25° C. Made on March 25th, 1904. Two loopfuls of the blood of a white rat were added to a tube of rabbit-blood-agar. The rat, whose blood was swarming with parasites, had been injected intra-peritoneally four days previously with blood taken from a Nagana rabbit. A drop of the culture, examined 24 hours later, was found to contain two or three active trypanosomes per field. On the fourth day several clumps of trypanosomes, containing as many as twenty or thirty trypanosomes, were found. The trypanosomes were irregularly arranged but possessed very active movements; they were somewhat granular in appearance. Pairs of trypanosomes, linked by their posterior extremities, were common. Free actively motile forms

[1] The strain of *Nagana*, possessed by this Laboratory, is well known. It was procured in 1896 from S. Africa by the Royal Society for investigations, which were made by Kanthack, Durham and Blandford ('98, p. 100), and has since then been maintained by the infection of rabbits. This strain was used by Laveran and Mesnil in their researches and by Novy and McNeal in their cultural experiments.

were not numerous. On the fifth day some of the trypanosomes were observed to contain vacuoles, which were very refractile in appearance and of a greenish tint. The vacuoles varied in size and number, they were usually situated in the anterior half of the organism. This culture never contained many colonies or free forms. At the end of three weeks the organisms were very granular in appearance and their movements were sluggish. On the twenty-eighth day, repeated attempts at subcultivation having in the meantime failed, 2 c.c. of fresh, defibrinated normal rabbit's blood were added to the culture, and 1 c.c. of this mixture was then added to a tube of freshly prepared rabbit-blood-agar, in order to obtain generation 2.

Generation 2. This culture (generation 2) was examined daily for eleven days, without finding any evidence of growth, but on making an examination on the twenty-fifth day, the culture was found to be swarming with organisms. Some of the colonies were of large size, containing about a hundred trypanosomes. This culture contained a few granular, motile forms on the thirty-ninth day.

Generation 3. Made with three loopfuls of culture of generation 2 on the twenty-sixth day. No living trypanosomes were found for the first three days, but on the fourth day a drop of the culture contained two small *rosettes* and several free forms. On the seventh day rosettes and free forms were numerous. The rosettes, which seldom contained more than twelve trypanosomes, were perfectly symmetrical in appearance, the flagella being directed outwards. The rosettes occasionally moved slowly across the field, but as a rule the active, lashing, movements of the individual organisms, composing the rosette, merely imparted to the latter a slight swaying movement. The rosettes were generally partly hidden by red blood corpuscles, which the trypanosomes seemed to attract around themselves. No rosettes were found on the eleventh day, but there were several large masses of trypanosomes which I shall describe subsequently as *colonies*. Free forms were also very numerous. On the fourteenth day there were few colonies, but trypanosomes, either free or in pairs, were numerous. This culture was alive on the twenty-first day, but the trypanosomes were not so numerous and many of them showed signs of degeneration. Three other subcultures, made at the same time as generation 3, from generation 2, were similar in character. Several attempts were made to subcultivate from generation 3, and from a parallel culture, but all failed[1].

[1] These subcultures were made just before taking a holiday; on my return in three weeks' time the original cultures were found to be dead.

Culture A. Made from the blood of a rabbit infected with Nagana. The blood of this rabbit was used for making the blood-agar as well as for inoculating the cultures with trypanosomes. The parasites were apparently all dead at the end of fourteen days, but, on examining the tube a month later, numerous dead clumps of trypanosomes and a few granular, slightly motile, colonies and free forms were found. Sub-cultures failed.

Culture B 1. Made from a white rat. A moderate growth was obtained at the end of three weeks. The culture showed signs of degeneration at the end of a month, and was quite dead in six weeks' time.

Culture B 2. Made from culture B 1, on its twenty-fourth day. A good growth was obtained at the end of three weeks, but unfortunately the culture became contaminated and further subcultivation was impossible.

Infection experiments with cultures of Tr. Brucei. Novy and McNeal (I. '04, p. 19) state that the virulence of the culture depends upon the temperature of cultivation, duration of growth, and possibly on the composition of the medium. They found that the exposure of a culture to a temperature of 34° C. for 2—6 days rendered the culture non-virulent, except in two instances (rats died on 14th day after injection with cultures incubated at 34° C. for five days). Most of their cultures, incubated at room temperature or at 25° C., were found to be virulent. The inoculations were made with cultures which varied in age from 7 to 22 days.

All the cultures of Nagana, which I have tested, have failed to produce infection in animals. Probably the cultures were too old; possibly in the case of Mouse no. 2 (see below) the dose was too small. The animals were injected intra-peritoneally with the culture fluid, which was mixed with a small quantity of normal salt solution. A list of these experiments is given below.

No. of animal	No. of generation	Amount injected	Age of culture	Result
Mouse 1	2	1 loopful	32 days	non-virulent
,, 2	3	1 loopful	15 ,,	,,
,, 3	B 1	3 loopfuls	25 ,,	,,
Rat 1	B 2	1 c.c.	28 ,,	,,

Morphology of Tr. Brucei in cultures.

(A) *Unstained preparations.* The organisms found in cultures of *Tr. Brucei* do not vary greatly in size and shape, and they resemble

closely the forms found in the blood. If a young culture is examined, the trypanosomes are found to possess very active movements. Sometimes they advance across the field moderately quickly, but their rate of movement is always much slower than that of the rat trypanosomes, whose flagella are longer and more rapid in action.

The undulating membrane is well-developed and its wave-like contractions are very clearly seen. The cultural forms are smaller than the trypanosomes found in the blood.

(B) *Stained preparations.* The organisms stain similarly to *Tr. Lewisi* but more deeply.

(1) *Body of the trypanosome.* This is usually bent or curved, its anterior extremity is pointed, the posterior end is usually slightly blunter. The protoplasm invariably contains a few deeply stained granules of a red or violet colour. The vacuoles, which were described on p. 39, are seen as clear circular spaces with sharply defined outlines in stained preparations. (See Figs. 5 and 6, Plate IV., and Figs. 3 and 4, Plate V.)

(2) *Nucleus.* The nucleus is round or oval in shape, and in older forms it breaks up into masses of chromatin, which are found distributed throughout the protoplasm of the cell.

(3) *Centrosome.* This structure is much smaller than in *Tr. Lewisi*, it is usually circular, but sometimes it is elongated. The centrosome stains a deep red or purple colour, it is sometimes difficult to distinguish it from the other granules. It is generally found close to a vacuole, sometimes it lies close to the nucleus, but it is nearly always *posterior* to the latter structure. (See *Tr. Lewisi*, p. 34.)

(4) *Flagellum.* The origin of this structure from the centrosome cannot always be seen. The flagellum takes a tortuous course along the free border of the undulating membrane, and projects for a short distance from the anterior extremity.

(5) *Colonies.* A description of the symmetrical "*rosettes*" has been already given on p. 39; it is more difficult to describe the larger masses of trypanosomes, composing the *colonies*. In the *living state* these structures are composed of closely packed, somewhat irregularly arranged, writhing masses of trypanosomes. It is rare to find colonies of a large size. The larger ones are not circular in outline as are colonies of *Tr. Lewisi*, but are generally elongated. Most of the flagella are directed in an outward direction. The active movements of the trypanosomes and the large glistening vacuoles, with which they are studded, give these colonies a singularly beautiful appearance.

It is not easy to obtain stained preparations in which the rosettes and colonies retain their form, since separation or massing together of the trypanosomes usually occurs in the process of making a film. Figs. 5 and 6, Plate IV. are from drawings of two rosettes (in stained films), which have retained their symmetry. Photographs of the same are given in Figs. 3 and 4, Plate V., for the purpose of comparison.

Measurements of the cultural forms of Tr. Brucei.

The following measurements were taken from stained preparations:—

Average dimensions (flagella included) } 18—23μ by 2·5—3·5μ (*26—27 by 1·5—2·5μ*).

Length of the flagella (free portion) 3—5μ.

Diameter of the vacuoles up to 1—2μ.

The dimensions printed in italics are those given by Laveran and Mesnil (1902, p. 19) for *Tr. Brucei* in the blood of rats.

Degeneration of the cultural forms of Tr. Brucei. The presence of numerous, large, highly refractile "globules" in the cultural forms of *Tr. Brucei* is attributed by Novy and McNeal to degeneration of the organisms owing to imperfections of the culture-medium. These globules become more numerous as the age of the culture advances. I have kept trypanosomes under observation in hanging drops for several hours without observing any alteration in the position or shape of their globules. The latter resist staining completely. Laveran and Mesnil (1904, p. 21) suggest that the globules, described by Novy and NcNeal, are of the same nature as the refringent, unstainable, granules found in *Tr. rotatorium*, and in *Tr. Lewisi*, if the latter be injected into the peritoneal cavity of a guinea-pig. The same authors attribute the vacuolation of *Tr. ugandense* (Castellani, 1903) to imperfect fixation of the trypanosomes, such as occurs in films made of cerebro-spinal fluid.

Mode of multiplication of Tr. Brucei. All observers agree that multiplication of this parasite occurs by longitudinal division. The process is similar to that occurring in *Tr. Lewisi*, but the division results in the formation of trypanosomes, which are approximately equal in size, and the flagellum splits along the whole or greater part of its length. The cultural forms multiply in the same way, but division is often incomplete, which accounts for the presence of so many paired forms in young cultures. Incomplete separation of the dividing forms also undoubtedly leads to the formation of the rosettes. The arrangement of the trypanosomes, composing the rosettes, with their flagella

directed outwards, is similar to that in the multiplication rosettes, found in the blood of rats infected with *Tr. Lewisi*. The question naturally arises, is the mode of formation identical in each instance? I have searched numerous preparations made from cultures of *Tr. Brucei* without finding any forms undergoing segmentation, and therefore conclude that the cultural, like the parasitic, forms of *Tr. Brucei* do not multiply by the process of segmentation.

Morphology of the cultural forms of Tr. Lewisi and Tr. Brucei summarised and contrasted.

(A) *Unstained preparations.*

Tr. Lewisi.	*Tr. Brucei.*
(1) Spindle-shaped or pear-shaped. Very variable in size, usually measure 3—5μ or 14—16μ (excluding the flagellum). Smaller and larger forms frequently seen.	Resemble the forms found in the blood of infected animals but are shorter and more pointed. More constant in shape and size than *Tr. Lewisi*, measure 15—20μ (excluding the flagellum).
(2) Move with great rapidity, generally in straight lines. The body of trypanosome is not curved or bent.	Movements are much slower and are generally of a wriggling character.
(3) *Protoplasm* is clear and homogeneous, rarely it contains a large single vacuole.	Protoplasm soon becomes slightly granular, invariably contains two or three large vacuoles.
(4) *Flagellum* very long and active, often quite rigid except at the point where it issues from the anterior extremity of the trypanosome.	Comparatively short but very active.
(5) *Undulating membrane* absent, unless it is developed in a very minute form at the base of the flagellum.	Well developed; its contractions are described by Novy and McNeal (1. '04, p. 27) as passing round the cell in a "spiral" direction.
(6) *Colonies* form large masses of cells, which are symmetrically arranged with their anterior extremities directed centrally. Huge colonies, visible macroscopically as small whitish granules, are formed by the coalescence of several colonies.	Colonies are of small size and are much less numerous. The younger colonies may present a symmetrical rosette-like appearance, the flagella being directed outwards. In the older colonies, the trypanosomes are closely packed together but are somewhat irregularly arranged. Secondary massing together of the colonies does not occur to any extent.
(7) Trypanosomes multiply rapidly. Cultures *swarm* with colonies and free forms and remain alive for three months or longer. Cultures retain their *virulence* for a long time.	Trypanosomes are never so numerous and degenerate rapidly; the culture being generally dead at the end of two months. Cultures rapidly lose their virulence, the time taken to do so depending upon the temperature of incubation.

(B) *Stained preparations.*

(8) *Protoplasm* stains a pale blue.	Protoplasm stains a deeper blue, and frequently contains deeply stained granules.
(9) *Centrosome*, usually rod-shaped and situated either at the margin of the nucleus or just anteriorly to it.	Centrosome much smaller, round or elongated and often difficult to distinguish from the other granules. Generally situated at the posterior end of the trypanosome.
(10) *Flagellum*, long and thick and projects for almost its entire length from the cell. Measures two to four times the length of the body of the trypanosome.	Flagellum is short and fine and can be traced backwards along the free border of the undulating membrane to end in the centrosome. Measures 3—5μ.

Since the above was written Novy, McNeal, and Hare (v. '04, pp. 1—8) announce that they have succeeded in cultivating the *Surra Trypanosome* of the Philippines. They found that the culture differed from those of *Nagana* in the following important respects: (1) The trypanosomes were larger, their average length was 25—35 μ, the flagella were very long. (2) The trypanosomes were very actively motile and moved either *backwards* or *forwards*. (3) The protoplasm of the anterior portion of the cell contained a large number of small "*granules or globules*" about 0·3—0·5 μ in diameter, of a yellowish or greenish tint; in forms dividing longitudinally, these globules were arranged in parallel lines, one row in each half of the dividing parasites. (4) There was an entire absence of rosettes and colonies.

The cultures (three in number) failed to subcultivate or to infect animals, although they remained alive for forty-eight to sixty-five days. The above mentioned investigators conclude therefore that *Surra* and *Nagana* are *distinct* forms of Trypanosomiasis, and they point out that Laveran and Mesnil and Nocard, as the result of immunising experiments, have expressed the same opinion.

Summary.

Tr. Lewisi has been cultivated by me for nine generations, the duration of cultivation extending over a period of nine months. The culture is still alive and is now in its tenth generation. A culture of *Tr. Brucei* was cultivated through three generations for a period slightly exceeding eighty days. These cultural experiments, however, in no way compare, in duration of time, with those of Novy and McNeal, who have cultivated *Tr. Lewisi* through twenty-six generations in two years, and *Tr. Brucei* through twenty-seven generations in eight months. Nagana trypanosomes degenerate rapidly in cultures and then become non-virulent, but a culture of *Tr. Lewisi* retains its infectivity

for rats for a long period. From the study of well stained preparations it has been found that the cultural forms of the rat trypanosome differ considerably from those of the adult parasite found in the blood. The chief points of difference are: (1) The trypanosome is exceedingly motile and is generally spindle-shaped. (2) The centrosome is placed anteriorly. (3) The flagellum is very long and active and has but a short intracellular course, in consequence of the position of the centrosome, from which it arises. (4) No undulating membrane is apparent. (5) The trypanosomes form colonies, which ultimately contain many thousands of individuals. The flagella of the latter are directed *centrally*. In rats, infected with cultures of *Tr. Lewisi*, the usual forms of parasite appear. The cultural forms of *Tr. Brucei* resemble the trypanosomes found in the blood, but are more active. The chief points of difference, viz. development of two or more vacuoles and numerous granules and sometimes the breaking up of the chromatin of the nucleus, are probably due to degeneration. The colonies, found in cultures of *Tr. Brucei*, consist of a comparatively small number of trypanosomes, which are arranged irregularly or with their flagella directed *peripherally*. The differences between cultures of *Tr. Lewisi* and *Tr. Brucei* are summarised on page 43.

In conclusion, I should like to acknowledge my indebtedness to Dr G. H. F. Nuttall, F.R.S., for his suggestion that I should repeat some of the interesting researches of Novy and McNeal. Dr Nuttall has very kindly examined many of my preparations, and he has placed at my disposal his very complete collection of published papers on the subject of Trypanosomiasis.

To Professor Sims Woodhead I am indebted for the many advantages which I have gained by working in the Pathological Laboratory, Cambridge.

The photographs were taken in this laboratory by Mr W. Mitchell, who has spared neither time nor trouble in his endeavours to obtain photographs suitable for reproduction. The various shades of red and blue, with which the organisms were stained, added considerably to the difficulties of micro-photography.

BIBLIOGRAPHY.

CASTELLANI (1903), Royal Society. Reports of the Sleeping-Sickness Commission, No. 2.

DUTTON and TODD (1903), First Report of the Trypanosomiasis Expedition to Senegambia (1902). *Thompson Yates, and Johnson Laboratories Report*, Vol. v.

KANTHACK, DURHAM and BLANDFORD (27. x. '98), On Nagana or Tsetse Fly Disease. *Proc. Roy. Soc.*, Vol. LXIV. p. 100.

KARTULIS (1891), *Centralbl. f. Backteriol.*, Bd. IX. p. 365.

LAVERAN and MESNIL (1901), Recherches morphologiques et expérimentales sur le Trypanosome des rats (*Tr. Lewisi*, Kent). *Ann. de l'Inst. Pasteur*, Vol. XV. p. 673.

—— (1902), Recherches morphologiques et expérimentales sur le Trypanosome du *Nagana* ou maladie de la mouche tsétsé. *Ann. de l'Inst. Pasteur*, Vol. XVI. p. 1.

—— (22. VI. '03), *Compt. rend. Acad. des Sc.*, Vol. CXXXVI. p. 1529.

—— (1904), Trypanosomes et Trypanosomiases, pp. 1—417, Paris, Masson et Cie.

LIGNIÈRES (1902), *Riv. Soc. med. Argent.*, Vol. X. pp. 112—114. (Cited by Laveran and Mesnil, 1904).

MCNEAL and NOVY (VI. '03), On the Cultivation of *Tr. Lewisi*. *Contrib. to Med. Research, dedic. to Victor Vaughan, Ann Arbor, Mich.*, pp. 549—577.

NOCARD (4. V. '01), *Compt. rend. Soc. de Biol.*, Vol. LIII. p. 466.

NOCARD, VALLÉE and CARRÉ (19. X. '03), *Compt. rend. Acad. des Sc.*, Vol. CXXXVII. p. 624.

NOVY and MCNEAL (21. XI. '03), On the Cultivation of *Tr. Brucei*. Reprint from the *Journal of the American Med. Assoc.*, Chicago, pp. 1266—1268.

—— (2. I. '04), On the Cultivation of *Tr. Brucei*. Reprint from *The Journal of Infectious Diseases*, Vol. I. No. 1.

NOVY, MCNEAL and HARE (28. V. '04), The Cultivation of the Surra Trypanosome of the Philippines. Reprint from *The Journal of the American Med. Assoc.*

RABINOWITSCH and KEMPNER (1899), Beitrag zur Kenntniss der Blutparasiten, speciell der Rattentrypanosomen. *Zeitschr. f. Hyg.*, Vol. XXX. p. 251.

SALMON and STILES (1902), Emergency Report on Surra. *Bull. No.* 42, *Bureau Animal Indust.*, U.S. Dept. Agric., Washington, pp. 1—152.

SCHARDINGER (1896), *Centralbl. f. Backteriol.*, Bd. XXII. p. 3.

SCHAUDINN (1904), *Arb. a. d. Kaiserl. Gesundteitstamte*, Bd. XX. pp. 387—439.

THEILER (1903), A New Trypanosoma and the Disease caused by it. *Journ. of Comp. Pathol. and Therapeutics*, Vol. XVI. pp. 194—216.

TSUJITANI (1898), *Centralbl. f. Backteriol.*, Bd. XXIV. p. 666.

WASIELEWSKI and SENN (1900), *Zeitschr. f. Hyg.*, Vol. XXXIII. p. 444.

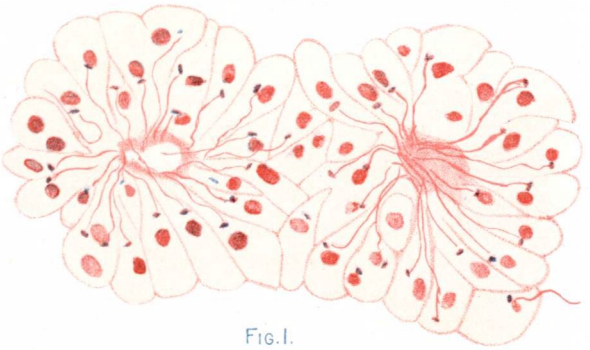

FIG. I.

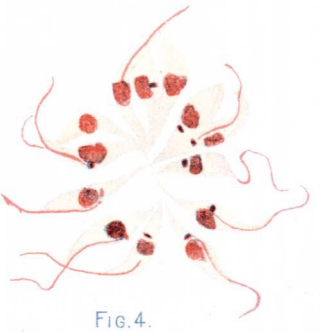

FIG. 4.

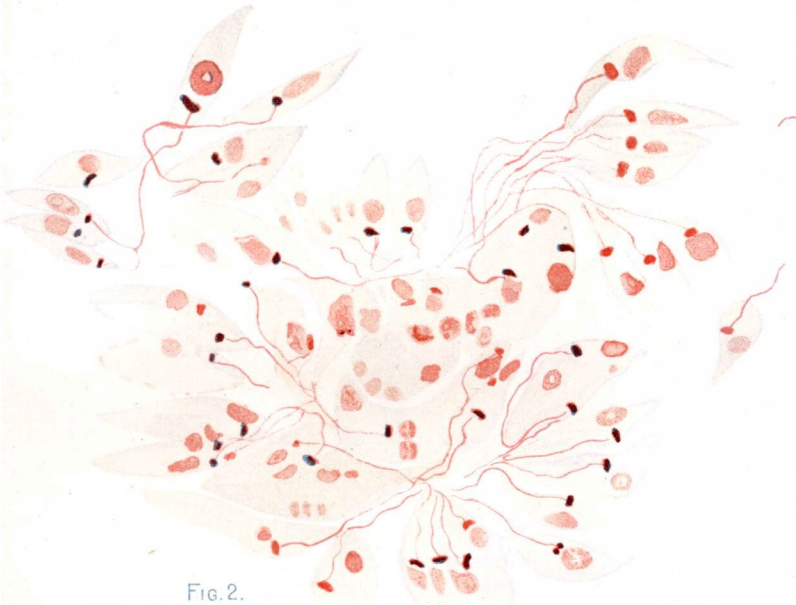

FIG. 2.

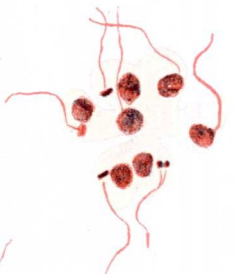

FIG. 3

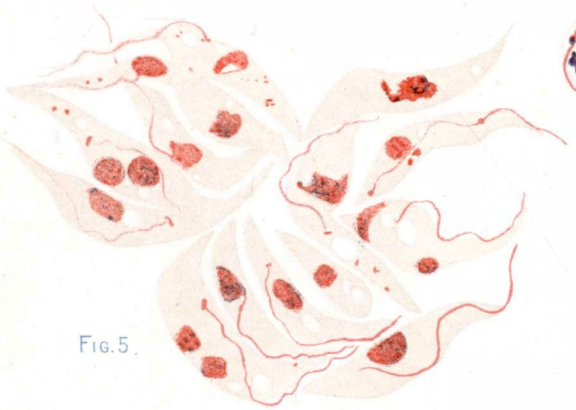

FIG. 5.

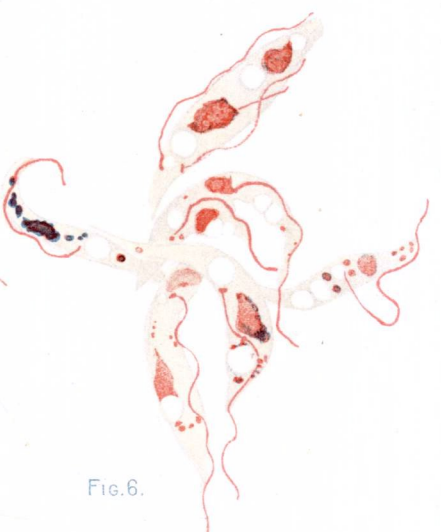

FIG. 6.

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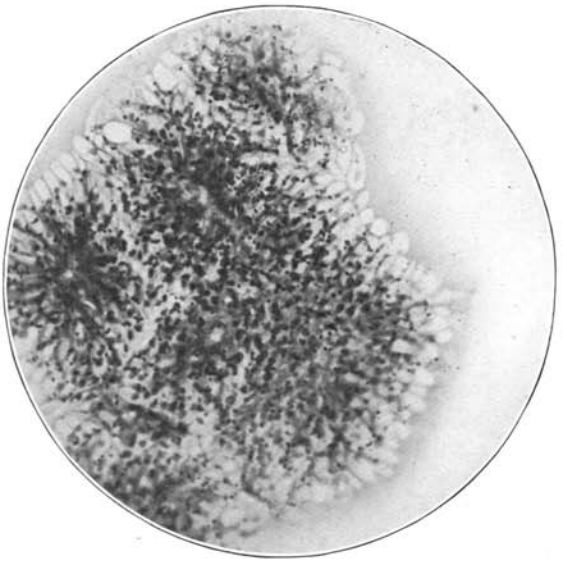

Fig. 2.

Fig. 1.

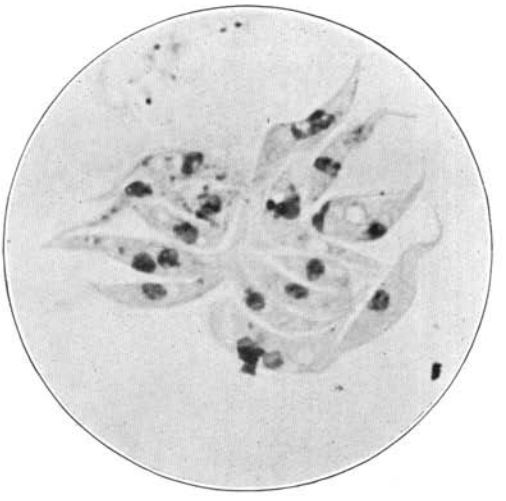

Fig. 3.

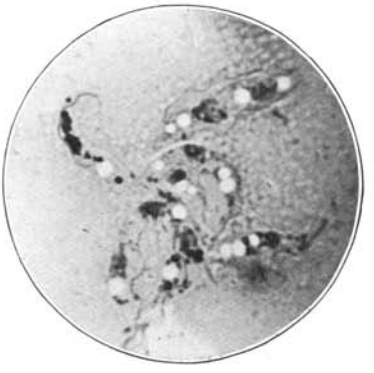

Fig. 4.

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EXPLANATION OF PLATES IV AND V.

Tr. Lewisi.

(From film-preparations stained by the method described on p. 33.)

Plate IV.

Fig. 1. Colony from a culture, 40 days old, of 2nd generation. ×1900 approx.
Fig. 2. Colony, much flattened out, from the same culture as above. ×1900 approx.
Fig. 3. Rosette, forming by process of segmentation. Blood-film preparation from a white rat, inoculated four days previously with a culture of *Tr. Lewisi*. ×1900 approx.
Fig. 4. Another rosette, more advanced stage of development. From the same preparation as that described under Fig. 3. ×1900.

Plate V.

Fig. 1. Young colony. From a culture, 25 days old, of 2nd generation. ×1250.
Fig. 2. Large colony. From the same preparation as that described under Plate I., Fig. 1. ×800.

Tr. Brucei.

Plate IV.

Fig. 5. Rosette, from culture, 8 days old, of 3rd generation. ×1900.
Fig. 6. Rosette, from a culture, 9 days old, of 3rd generation. Period of cultivation extended over 62 days. ×1500.

Plate V.

Fig. 3. Same rosette as that described under Plate IV., Fig. 5. ×1250.
Fig. 4. Same rosette as that described under Plate IV., Fig. 6. ×1000.