Notes on Toxoplasma gondii.

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(Communicated by Prof. E. A. Minchin, F.R.S. Received April 12,—Read May 29, 1913.)

[Plate 9.]

The genus *Toxoplasma* was founded by Nicolle and Manceaux (3, 4, 5) for *T. gondii*, a species discovered by them parasitic in the gondi (*Ctenodactylus gondii*) in Tunisia. When first discovered the parasite was identified as a new species of *Leishmania*. Further investigation showed, however, that its resemblance to the true *Leishmania* type was quite superficial, since it neither possessed a kinetonucleus nor gave rise to flagellated forms in cultures, consequently it was made the type of the new genus *Toxoplasma*, of which the affinities and systematic position are at present very doubtful. Since then other species of *Toxoplasma* have been described from rabbits in Brazil (*T. cuniculi*, Carini), from dogs in Italy and Brazil (*T. canis*, Mello), from moles in Japan (*T. talpæ*, Prowazek), and from pigeons in Brazil (*T. columbæ*, Yakimoff and Kohl-Yakimoff [10]).

Very recently Nicolle and Conor (6) have given an account of the parasitism of *T. gondii* and the reactions of susceptible experimental hosts.

In January of this year Dr. Nicolle most courteously presented Prof. Minchin with some mice, two of which had been inoculated from an infected pigeon at Tunis. These were brought to the Lister Institute and handed over to me for study. I am indebted to Prof. Minchin not only for the privilege of examining so interesting a parasite but also for much kind help and advice.

The two infected mice died shortly after their arrival in England: one, in fact, on the night after it arrived in London. In order to preserve the strain of the parasites, other mice or pigeons were inoculated by Prof. Minchin from those that died. I have thus been able to make *post-mortem* examinations of animals that had died recently from the effects of the parasite or had been freshly killed.

Although I propose in this memoir to deal chiefly with the minute structure and reproduction of *Toxoplasma*, which has never yet been studied by cytological methods, I may also refer briefly to some points in the occurrence of the parasites and their effects on the experimental hosts that have come under my observation in the course of the work.

Course of the Infection in Mice and the Distribution of the Parasite in the Tissues.

Mice are very susceptible to the effects of the parasites, which prove fatal in from 5 to 15 days after intraperitoneal inoculation. The symptoms are: (1) an increased rate of respiration until the last 12 hours or so, when breathing becomes slow and laboured; (2) a general loss of the senses, especially sight—the mice appearing to be blind for the last day or two; (3) general lethargy—food, however, is taken in many cases almost as usual until nearly the end.

The individuals showed many *post-mortem* differences, which will be considered under the different tissues.

(1) *Peritoneal Fluid.*—In some cases the peritoneal cavity is full of a viscid, slightly cloudy fluid containing numerous parasites, both free and intracellular—in Mice C and D (see below) this was the case and these two were undoubtedly the best infected specimens—Mouse F, however, which had an equally large amount of fluid, seemed to have nothing like the number of parasites, not more, in fact, than A, in which there was hardly any peritoneal fluid. It will be noticed, too, that in both A and F the course of infection lasted seven days.

Other anomalous cases will be noticed among the results of experiments tabulated below, from which it would seem that the quantity of peritoneal fluid cannot always be correlated with the number of parasites present, nor is the rapidity with which they prove lethal necessarily proportional to their number. On the other hand D had, perhaps, the best infection, and it was the most rapidly fatal, whereas B, in which the disease lasted 12 days, was found on *post-mortem* examination to have only a very poor infection.

The parasites in well infected animals are to be found in numbers both free in the fluid and enclosed in the cells floating in it. Of the leucocytes they are nearly always mononuclears (Plate 9, fig. 1) that are affected; only occasionally has a polymorphonuclear been found to contain one or two parasites. In other cases the parasites are embedded in, or attached to, cellular debris (figs. 2, *a–c*); the origin of these masses of debris is sometimes difficult to determine—they are referred to by Nicolle (5, p. 98) as "gangues." Still more numerous, however, are the parasites in endothelial cells (macrophages) which have evidently become detached from the peritoneum and float freely in the fluid (figs. 3 and 4).

(2) *Mesenteries.*—Finding that detached endothelial cells were so often infected with parasites, I was led to examine the mesenteries themselves, with the result that the endothelial cells forming these serous membranes were found in many cases to be packed with parasites (figs. 5 and 6).

Major S. R. Christophers tells me that the serous membranes have not, so far as he is aware, been examined in cases of Kala Azar, and he suggests that the systemic parasite, *Leishmania donovani*, may possibly be found to infect the membranes and peritoneal fluid in patients suffering from this disease also, since the two parasites seem to be similar in so many other ways.

In preparations of the stretched omentum, stained with silver nitrate and the other reagents mentioned below, large pavement cells may be seen crowded with as many as sixty or seventy parasites (fig. 6), other cells may have only one or a few toxoplasms (fig. 5). In the latter case the nucleus of the host-cell sometimes retains its normal oval contour with one or two distinct nucleoli (fig. 5, *a*), but in all those enclosing more than two or three parasites the nucleus has become more or less rounded, with the chromatin in several blotches, giving a decidedly necrotic appearance. In fig. 5 it will be seen that the central cell (*a*) is uninfected and normal; the right-hand one (*b*) contains two parasites and already the nucleolus is beginning to break up. In the left-hand cell (*c*) the degeneration of the nucleus has advanced farther still, though not so far as the nuclei of the cells represented in figs. 4 and 6. In these, as in many other cases, two such necrotic nuclei are present, which fact seems to suggest that after nuclear division had taken place, the infected cell had not sufficient vitality for the division of the cytoplasm.

The long, narrow, endothelial cells, which in many cases show branching ends, are also very often full of parasites, and although the nucleus in these may remain more or less oval, it could not be mistaken for that of a normal cell, owing to its general necrotic appearance. In transverse sections of the mesenteries parasites could be distinguished not only in the flat endothelial pavement cells forming the serous membrane, but also in the connective tissue corpuscles of the subserous areolar tissue. None, however, was observed free in the lymphatics or capillary blood-vessels, nor in their endothelial linings.

In mice, such as B, I, and J, described below, which were found with only very few parasites in the peritoneal fluid, the mesenteries also seemed to be destitute of them, and in such cases very few toxoplasms could be found anywhere in the body.

(3) *Liver.*—The parasites here are less numerous than in the peritoneal fluid. When present they are frequently seen to be dividing, and may be free (figs. 13–15), or in the mononuclear leucocytes, never apparently in hepatic cells. In Mouse L, in which the infection lasted 15 days, the liver had become pale and friable, and was much hypertrophied.

(4) *Spleen.*—This organ generally has fewer parasites than the liver. They may be included in mononuclears or be free.

(5) *Blood.*—The peripheral blood does not appear to contain parasites. It has been tested in some cases during life.

The heart-blood was found to contain a few parasites in the two cases examined.

(6) *Kidney.*—No parasites were found in this organ in the one case examined.

(7) *Lungs* and pleural fluid contain a few toxoplasms, at any rate in some cases.

(8) *Aqueous Humour and Cornea.*—No parasites could be found in the two cases examined (Mice C and J).

(9) *Brain.*—No parasites were found in Mouse J.

(10) *Bone Marrow.*—No parasites were found in Mouse L.

(1) *Experimental Infection of Mice.**

Mice A and B were inoculated from the liver of a slightly infected pigeon which was killed at Tunis on January 10. In all cases inoculation was intra-peritoneal from peritoneal fluid unless otherwise stated.

Mouse A.—Died January 17. Course of infection seven days.

Only a little peritoneal fluid was present, but a number of parasites were found, both free and intracellular. The liver contained a few, chiefly intra-mononuclear parasites.

Mouse B.—Died January 22. Course of infection 12 days.

Very little peritoneal fluid and only a few parasites. Liver and spleen scarcely any parasites.

Mouse C.—Inoculated January 17 from A. Died January 25. Course of infection eight days.

Large amount of peritoneal fluid and many parasites here and in mesenteries. Left eye opaque, but no parasites found in aqueous humour or cornea, and lens appeared to be normal.

Mouse D.—Inoculated January 25 from C. Died January 30. Course of infection five days.

Large amount of peritoneal fluid and numerous parasites in this and the mesentery.

Mouse E.—Inoculated January 30 from D. Died February 5. Course of infection six days.

Not much peritoneal fluid nor very many parasites.

Mouse F.—Inoculated February 5 from E. Died February 12. Course of infection seven days.

Large amount of peritoneal fluid, which was, however, poor in parasites.

* These inoculations were performed by Prof. Minchin under his licence.

Mouse H.—Inoculated February 12 from F. Died February 20. Course of infection eight days.

Very little peritoneal fluid, and this was poor in parasites, so also the mesentery. Liver, spleen and heart-blood contained a fair number.

Mouse I (with three legs only).—Inoculated February 20 from H. Found dying January 26 and chloroformed. Course of infection six days.

Very little peritoneal fluid and a very poor infection. Lungs and liver found to be infected with a bacillus and also a diplococcus. The presence of the latter would suggest that the mouse was dying of pneumonia. Inoculation of peritoneal fluid into another mouse gave no result.

Mouse J.—Inoculated February 20 from the liver of H. Died March 3. Course of infection 11 days.

Only a little peritoneal fluid with a few parasites. The mesentery also only contained a few. In the pleural fluid occasional specimens were found, as also in the lungs, heart-blood, and liver.

Mouse L.—Inoculated March 3 from J. Died March 18. Course of infection 15 days.

A quantity of fat present and the animal seemed generally well nourished. A fair amount of peritoneal fluid, but it only contained a few parasites. Liver hypertrophied and of a somewhat friable consistency, but it only contained a small number of parasites and none could be found in the bone marrow.

Mouse G was fed February 5, 12, and 20 on material infected with parasites, but was apparently none the worse on March 19.

Conclusions—

(1) Infection would not seem to take place in nature by means of the alimentary canal unless the parasite may possibly be swallowed in a form different from that in the vertebrate host.

(2) The disease ran its longest course in Mice B, J, and L, which were found in *post-mortem* examination to have only a few parasites. If the rapidity with which the poorly infected Mouse I succumbed be ascribed in part to its pulmonary bacterial infection and general unhealthy condition, then it would seem that the length of the course of infection is approximately inversely proportional to the number of parasites in the whole body and that the death of the host may be due to their cumulative toxic action.

(2) *Pigeons.*

Pigeon 1.—Inoculated January 22. Intraperitoneum from peritoneal fluid of Mouse B. No effect.

Pigeon 2.—Inoculated January 30. Intraperitoneum from peritoneal fluid of Mouse D. February 26, peripheral blood tested, no parasites found.

March 19, appeared in perfectly normal health.

I cannot account for this pigeon not becoming infected after being inoculated with the peritoneal fluid of Mouse D, which contained numerous parasites.

[*June* 19.—I understand from Dr. Nicolle that he has also found that pigeons cannot be infected by inoculation of the virus after passing through mice, though he has succeeded in infecting them easily directly from gondi.]

Technique.

Many smears were stained with Giemsa, and the presence of parasites was easily tested in this way, although, as is well known, this stain cannot be relied upon for cytological detail. On the whole iron hæmatoxylin was found to be by far the best nuclear stain. Delafield's hæmatoxylin also gave good results. Twort's stain, borax carmine, paracarmine, Mayer's acid hæmalum and Mann's hæmatein were used with less success. Double staining was found to be advisable in all cases. Orange G and eosin were quite satisfactory for this purpose. No differential staining of the cytoplasm was effected by using mixtures such as licht-grün and picric acid or picronigrosin; both of these were, however, useful for the stretched omentum. Another useful mixture for sections was eosin and licht-grün made according to Chatton's formula (2, p. 254), which is a modification of Prénant's. The cytoplasm of the parasite took up the eosin only, so apparently there are no reserve food-particles or other green-staining inclusions.

When fixed by osmic acid vapour and absolute alcohol for Giemsa staining, the parasites, as usual, appear much larger (figs. 7 and 8) than when fixed by the wet methods generally employed. For the latter Maier's fluid gave very good results—slightly better perhaps than Flemming's fluid or a mixture of corrosive sublimate and acetic acid.

Morphology of the Parasite.

The living parasites are non-motile, but seem to be capable of slightly altering their shapes. A specimen drawn with a camera lucida at intervals of 5 or 10 minutes is shown in figs. 2, *a–e*. It appeared to be trying to free itself from a mass of cellular debris. I would not like to say for certain, however, that it was really changing its shape, for the apparent differences in appearance may possibly have been due to slight invisible currents in the medium causing the parasite to be viewed from different aspects. I have also observed them apparently bending in the middle and turning over.

In size, as can be seen from the figures, they agree very closely with the measurements given by Nicolle (5, p. 99) for the average parasite from the gondi, namely, 5–5·5μ in length by 3–4μ in breadth.

The nucleus is generally clearly visible as a rounded region, clearer than the rest of the body and somewhere near its centre (figs. 9 and 10). Fig. 11 shows a dividing form with two nuclei.

In some specimens definite round refringent granules are to be seen, sometimes only one as in fig. 9, but never in large numbers. These will be referred to again later.

In preparations stained by one of the exact cytological methods it can easily be seen that the nucleus is of the protokaryon type, that is, it consists of a sharply defined karyosome suspended in a clear vesicle (fig. 12). The nucleus is probably bounded by a membrane, which is, however, in no case distinct, and only occasionally is there any peripheral chromatin (fig. 13). The karyosome, no doubt, encloses a centriole, since a centrodesmose is formed during fission, as will be described later, but it has not been found possible to differentiate the staining of the small karyosome in order to make the centriole apparent.

In the alveolar cytoplasm there are often fairly large vacuoles, and the refringent granules seen in living specimens take up chromatin stains very readily, and are probably, I think, of the nature of reserve chromatin, or volutin, as described by Reichenow (6, pp. 328–331) for *Hæmogregarina stepanowi*. In some cases they have almost exactly the size of the karyosome, and since they may occur quite close to the nucleus (figs. 7, 14, 15, 17, and 22), they are apt to be somewhat confusing.

The parasites generally lie in distinct vacuoles in the protoplasm of the host-cell (figs. 3–5); the formation of these might possibly be attributed to the reaction on the part of the cell by which it throws out some sort of secretion round the parasite, as suggested by Row (7, p. 749) in the case of *Leishmania tropica*, but it seems more likely to indicate a destructive liquefying action by the parasite on the protoplasm of the host-cell. This latter explanation would account for the fact that cells infected with many parasites are in an advanced state of necrosis.

Free parasites may be of different sizes and are frequently found undergoing fission. Intracellular forms often appear to have divided repeatedly in rapid succession, giving rise to forms which are much reduced in size (fig. 4, *s*). Those nearer the boundary of the cell seem to break away at intervals as shown in this figure, and all that is seen to remain of some cells is a degenerating nucleus with a fringe of cytoplasm.

Multiplication of the Parasite.

Toxoplasma divides by the simplest form of binary fission. The karyosome elongates and becomes dumb-bell shaped (figs. 16 and 17). The two daughter-karyosomes then move apart from one another, remaining connected by a short centrodesmose (figs. 18 and 19). This soon appears to snap, for it has disappeared by the time the stages represented in figs. 20 and 21 are reached. The vesicle is then constricted off and the two daughter nuclei separate (figs. 22–25).

Division of the body is usually longitudinal, but may sometimes apparently be transverse, or oblique (fig. 24). Fig. 22 shows two daughter-individuals which have evidently just been formed by longitudinal division, and one appears to be again dividing longitudinally, the other transversely.

In some infected cells, perhaps owing to the fact that the parasites can divide in different directions, compact more or less spherical masses of 20 or more parasites may be produced which have something the appearance of a cyst. A spherical mass of this kind is seen to be forming at *s* in fig. 6 and probably also in figs. 4 and 5. The first of these is, however, scarcely half the size of the masses seen in many infected cells. Nicolle (5, p. 99) suggests that this appearance may have led Splendore (9) to interpret erroneously similar masses as cysts in the rabbit. In the schizogony, recently described by Yakimoff and Kohl-Yakimoff (10, p. 202) as taking place in free or intracellular forms, multiple fission into 32 or more may apparently take place, but it is impossible to make out the details of the process from their figures, made from Giemsa preparations.

Attempts to make Cultures of the Parasite.

Attempts were made to cultivate the parasite on agar plates, on blood agar, and in blood serum, also by adding to some peritoneal fluid a small percentage of 50-per-cent. dextrose as recommended by Bass (1) for the cultivation of the Malaria parasite, but in no case could any different form be produced.

This seems to be all that can be done here in connection with *Toxoplasma*, but I hope to carry on this work next month in the desert of South Tunisia, which is the most northerly haunt of its natural host—the gondi—and there to investigate its method of transmission and life-history.

[*June* 19.—In the above I have purposely refrained from discussing the affinities of *Toxoplasma* until such time as its life-history should be known, for until then its systematic position must remain uncertain. The parasite

is, however, to all appearances a true Protozoon. Since Roche-Lima* has claimed to show that *Histoplasma* is a yeast-like organism, the question naturally arises as to the possibility of *Toxoplasma* being also related to the Blastomycetes. That such is not the case, however, is, I think, sufficiently proved by the characteristics described above, such, for example, as: (1) the constant appearance of the nucleus; (2) the absence of a distinct refringent membrane round the parasite; (3) the fact that it does not grow in cultures; and (4) that no sign of gemmation has been observed, the parasite generally dividing by longitudinal fission. Beer-wort gelatine was used for culture experiments in addition to the media mentioned above, but in no case could the *Toxoplasma* be made to grow, neither would they retain any colour when preparations were stained by Gram's method.

Nicolle and Conor (6) in their recent paper, which I saw for the first time in Tunis on my way to the desert, gave some evidence of the fact that *Toxoplasma* causes only a seasonal disease in the gondi. This I was able to confirm, for during April, when I was at Matmata (the place from which the disease was originally recorded by Dr. Nicolle), I could find no trace of *Toxoplasma*, although I examined 55 gondi and numerous other indigenous animals, including rats, mice, shrews, many birds, such as finches, pigeons and eagles, also a palm lizard, snakes, and frogs. I also carefully examined the ectoparasites of the gondi, these being in nearly every case the hexapod larvæ of a mite (*Trombidium* ?) clustered together, forming yellow masses in the ears, and ticks which Prof. Nuttall and Mr. Warburton have kindly identified for me as *Rhipicephalus* (*Pterygodes*) *fulvus*, Neumann. The latter were all in the nymph stage, but some have already metamorphosed since I brought them to England, and I am now hoping to make the adults feed on mice, guinea-pigs, or rabbits infected with *Toxoplasma*. Prof. Mesnil has most kindly sent me the virus for these experiments from Paris. In neither of the natural ectoparasites of the gondi could I discover anything that could be recognised as *Toxoplasma*, nor do I think that the parasite could be present in an unrecognisable form, for no results have been obtained by inoculating their contents into mice, gondi, and a pigeon. Dr. Nicolle most kindly performed these inoculations for me, and has kept the pigeon, gondi, and some of the mice under observation at the Pasteur Institute, Tunis; owing to the great delicacy of the gondi, it would not have been safe to risk bringing them to London.

I am very much indebted to Dr. Nicolle also for most kindly making excellent arrangements for my visit to the desert, and for providing me with

* 'Centr. für Bakter.,' 1913, Abt. 1, vol. 67, pp. 233–249.

plentiful reagents and apparatus for my work there. I should also like to thank the French military authorities for their courtesy and kindness during my stay at the Matmata Fort, where they gave me all possible assistance.]

EXPLANATION OF PLATE 9.

Unless otherwise stated, the preparations were stained with iron hæmatoxylin and counterstained with licht-grün and picric acid.

Fig. 1.—Mononuclear leucocyte enclosing several toxoplasms. × 2000.

Fig. 2.—Living specimen drawn at intervals of 5 or 10 minutes. × 2000. *a*, *b*, still attached to cellular debris ; *c–e*, after becoming free.

Fig. 3.—Detached endothelial cell containing two parasites. × 1000.

Fig. 4.—Detached endothelial cell with two nuclei and several parasites, some of which are being set free. × 1000.

Fig. 5.—Three endothelial cells of the serous membrane. × 1000. *a*, normal cell ; *b*, cell with two parasites and nucleolus already beginning to break up ; *c*, cell with a further degenerated nucleus, and several rapidly dividing parasites forming a mass at *s*.

Fig. 6.—Cell from the serous membrane of the omentum with more than 60 parasites, some forming a compact mass at *s*. × 1000.

Fig. 7.—A toxoplasm from the liver of Mouse H, showing chromatoid masses in its cytoplasm. × 2000. Stained Giemsa.

Fig. 8.—Free dividing form from liver. × 3000. Stained Giemsa.

Figs. 9 and 10.—Living specimens from peritoneal fluid with one or more refringent granules in cytoplasm. × 2000.

Fig. 11.—Living specimen with two nuclei from peritoneal fluid. × 2000.

Fig. 12.—Intracellular parasites from a transverse section of omentum. × 3000. Stained iron hæmatoxylin, eosin, and licht-grün.

Fig. 13.—Form with some peripheral chromatin in the nucleus. × 3000. Stained iron hæmatoxylin and orange G.

Figs. 14 and 15.—Recently-divided forms with extranuclear chromatoid masses probably consisting of volutin. × 2000. 14. Stained iron hæmatoxylin and fuchsin S.

Figs. 16–25.—Free parasites showing binary fission. × 3000. 25. Stained iron hæmatoxylin and orange G.

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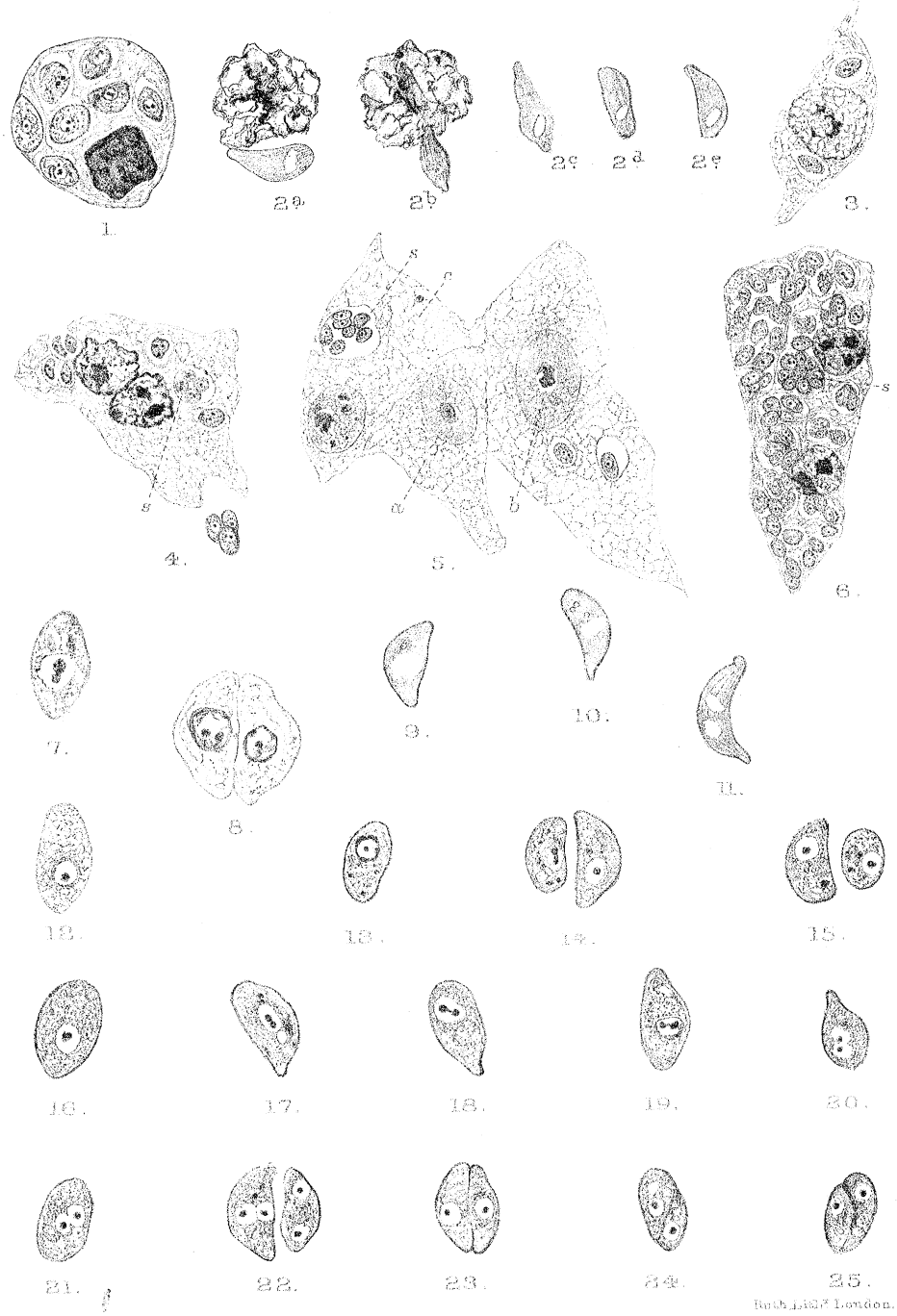

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The Growth and Sporulation of the Benign and Malignant Tertian Malarial Parasites in the Culture Tube and in the Human Host.

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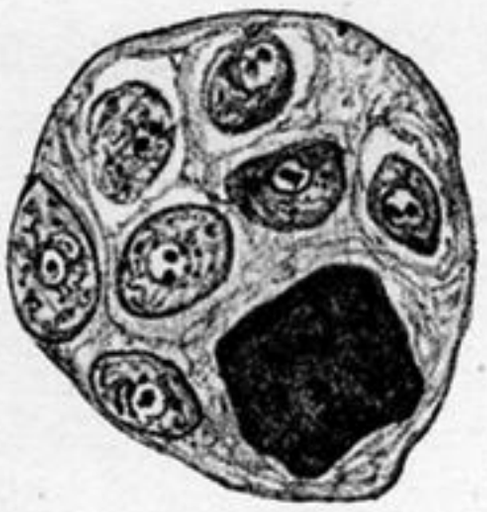
[PLATE 10.]

Prefatory Note.

Researches on the cultivation of the parasites of malaria in Liverpool were commenced some time ago at my suggestion by Dr. Sinton, and then, with better success, by Drs. J. G. Thomson and McLellan, and by Dr. D. Thomson. We are greatly obliged to Sir Edwin Durning-Lawrence, Bart., for giving us the services of Dr. J. G. Thomson for this important enquiry.—RONALD ROSS, 21st May, 1913.

Introduction.

The successful cultivation of malarial parasites was first announced by Bass and Johns (1912). Since then several workers, Thomson and McLellan (1912), Thomson, J. G., and Thomson, D. (1913), and Ziemann (1913), have successfully repeated these cultivation experiments. This achievement has led the way to new discoveries regarding the malarial parasite, and suggests that it may be possible to cultivate *in vitro* any protozoal parasite, however specialised it may be.



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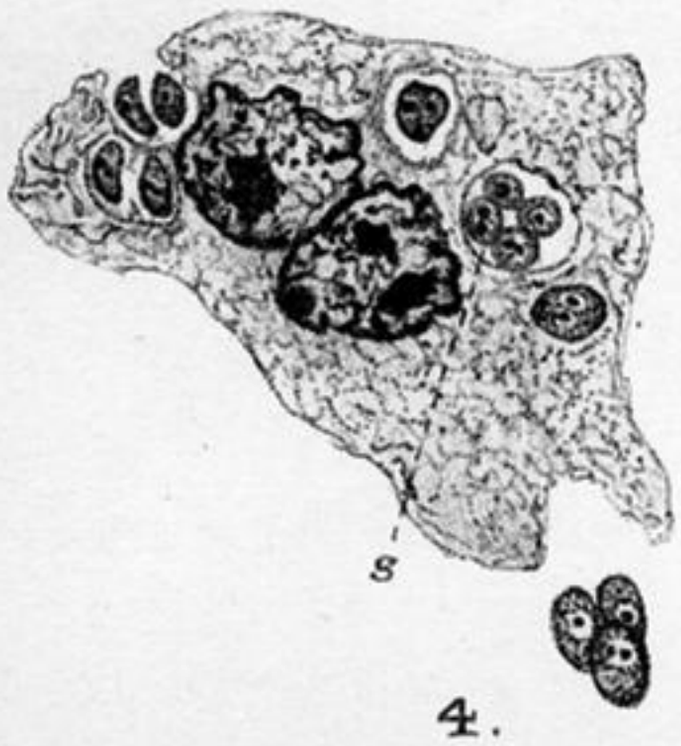

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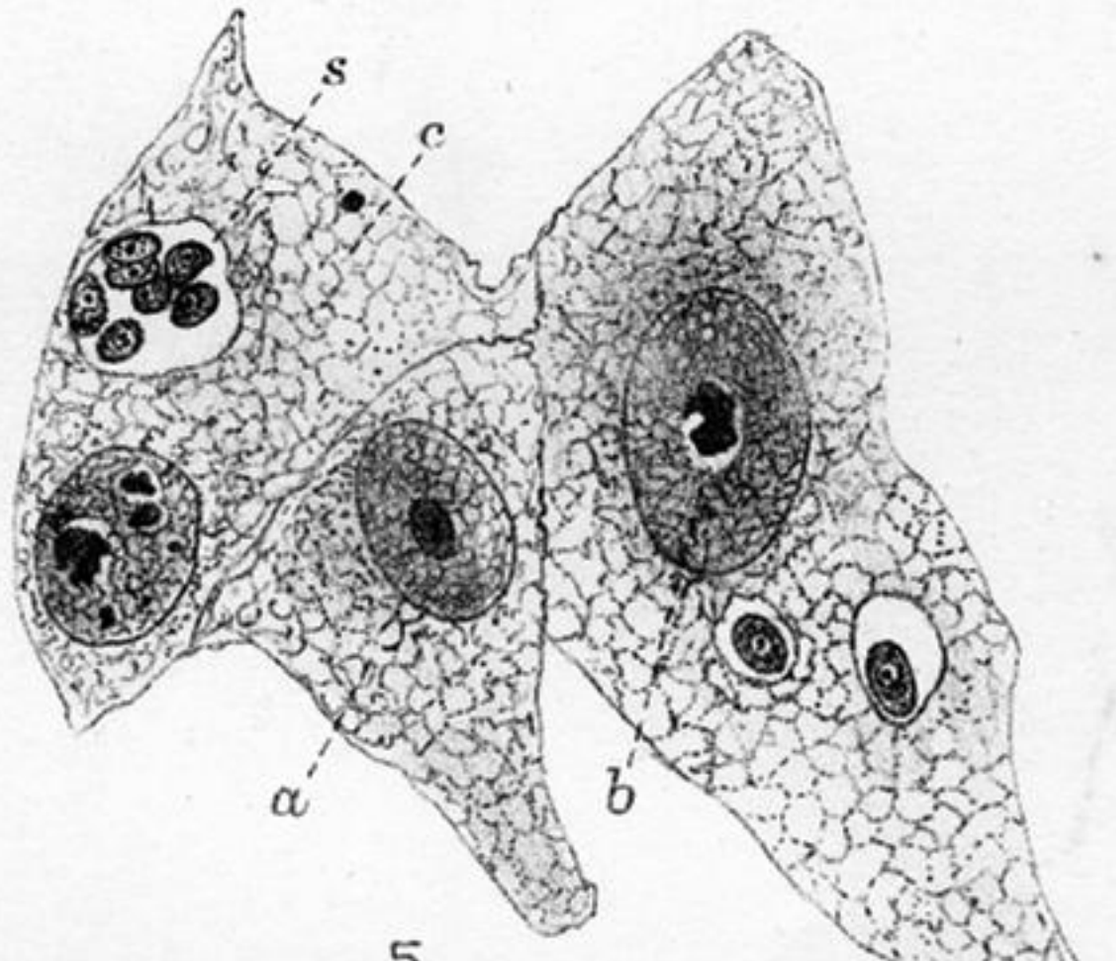

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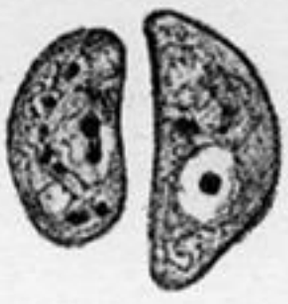

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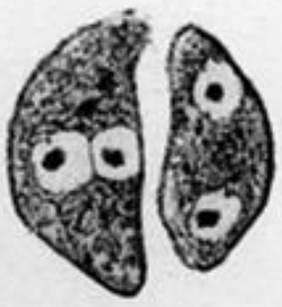

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