Further Work on the Development of the Hepatomonas of Kala-Azar and Cachexial Fever from Leishman-Donovan Bodies.

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[Plate 7.]

In 1903 Lieutenant-Colonel Leishman, R.A.M.C., described certain bodies in the spleen of a fatal case of chronic fever in a soldier invalided home from near Calcutta, which he considered to be degenerate trypanosomes, on account of their resemblance to the breaking-up dead trypanosomes found in the spleens of rats 48 hours after death, and he therefore suggested that trypanosomes might be present during life in this class of fever. Major Donovan, I.M.S., (2) of Madras, however, very shortly after showed that Leishman's surmise was not correct, as he found similar bodies to those described by Leishman in fresh blood obtained by puncturing the spleen during life, but no trace of trypanosomes; and after examining Donovan's specimens, M. Laveran (3) pronounced the parasite to be a Piroplasma, and suggested the name *Piroplasma Donovani* for them.

Donovan (4) also claims to have found the parasites in the red corpuscles of the peripheral blood, but his coloured illustrations of them very closely resemble ring-parasites of malaria, and have only one chromatine body, and his statements in this respect have not been confirmed by any other observer. Ross (5) suggested that the parasite probably belonged to a new genus, and proposed to call it *Leishmania Donovani*, and Manson and Nuttall also favoured the view that it is a distinct genus. In the following year, 1904, on my return to India from leave, I commenced an investigation of the subject, with a view to finding further stages of the life history of the parasite which might throw light on its true nature and classification. In the meantime Lieutenant Christophers, I.M.S., had been placed on special duty by the Government of India to investigate the subject, and after making a careful study of the parasite in different tissues of the body, he suggested that they might be spores of a microspordium. (6)

My first endeavour was to find some method of keeping the parasite alive outside the human body, and after a number of trials success was obtained by preserving spleen blood containing the parasites under sterile conditions in a cold incubator, preferably at about 22° C. For this purpose the fresh blood obtained by spleen puncture during life was placed in small sterile

test-tubes containing a few drops of 2 to 5 per cent. citrate of soda in normal salt solution, in order to prevent clotting. Under these conditions not only did the parasites remain alive for many days, but they also multiplied very rapidly, became much enlarged, and after about three days some of them developed into elongated flagellated bodies, which I took to be a stage in the development of a trypanosome, although no undulating membrane was yet present. This discovery was announced in the 'Lancet' of July 23rd, 1904, a few uncoloured illustrations were published in September, (7) and a fuller paper tracing the stages of the development day by day, with a coloured plate, in November (8) of the same year. Confirmation of my discovery was first furnished by my assistant, Dr. G. C. Chatterjee, (9) working in my own laboratory, and next independently by Lieutenant Christophers, (10) working in Madras. Thirdly, Captain Statham (11) and Lieutenant-Colonel Leishman also obtained the development, and published their account of it, March, 1905.

During the past year I have made a large number of experiments on the conditions affecting the development of the parasites outside the human body, with a view to obtaining a clue to the natural mode of infection, and early in the present year I published a summary of the results obtained. The most important conclusions arrived at were, that sterility is essential to the continued development, and that flagellation takes place much more uniformly and regularly if the citrated spleen blood is faintly acidified with citric acid: facts which strongly point to the stomach of some blood sucking insect as the natural place of development of the parasite outside the body, and I gave some clinical reasons for considering the common bed bug to be the most likely conveyer of the disease. So much more abundant development of flagellated stages has recently been obtained by the use of acidified blood medium, that I have been able to make a more satisfactory study of the exact mode of development, and to come to a definite conclusion regarding the ultimate stage it reaches, and therefore propose to describe and illustrate these later stages more fully than in my previous papers, and to briefly discuss the bearing of the conditions affecting the development of the flagellated stage of the parasite on the probable mode of infection of the disease.

Stages of Development of the Parasites observed in Acidified Citrated Blood.

In the first place, the development in acidified blood is much more uniform than that obtained by the previous method, so that instead of finding all stages present after three or four days, with a great preponderance

of the smaller oval forms, and but few flagellated ones as in alkaline blood, in the acid medium the great majority of the parasites will be found in nearly the same stage on any given day, and nearly all become flagellated after a few days. The sequence of events during the first two days is the same as I have already described, (8) and they are well shown in the first two lines of the accompanying plate—all the figures in which have been drawn to the uniform scale of 1500 diameters magnification, with the aid of a camera lucida. Line I shows the parasites seen in a film of spleen blood made at the time it was obtained, and consequently before any development had taken place. After incubation for two days at 22° C. the forms shown in Line II were present, figs. 1 and 2 showing considerable enlargement, especially of the macronucleus and protoplasm of the body. Figs. 3 and 4 also show the earliest appearance of the eosin-staining body, which is represented as a clear space in the drawings, but is of a rosy-pink colour with Romanosky's stain, and quite distinct from the vacuoles, the latter being indicated by the more lightly shaded portions of the protoplasm. It will also be noted that from the first the micronucleus, or blepharoplast, is closely attached to the eosin body (called by Leishman "flagellar body").

Further, on the second day in this acid culture a few of the early flagellated forms shown in figs. 7 and 8 of Line II were also seen, although they do not usually appear in alkaline cultures until at least the third day, while just antecedent to this stage are the forms shown in figs. 5 and 6 of the same line, illustrating commencing elongation and division by fission, and it will be observed that in these the eosin body is passing up to the anterior end of the organism from which the flagellum arises, and is carrying the micronucleus with it. In my earlier description I suggested that the double elongated forms shown in fig. 6 of this line might possibly represent a form of conjugation preparatory to the development of the flagellated stage of the organism, but further study of a much larger amount of material has convinced me that they are only fission forms, as I have been unable to make out any reduction in the number of chromosomes in the macronucleus during the process.

The Mode of Division of the Flagellated Forms and the Formation of Rosettes.

In Line III of the plate are represented the different stages of division of newly formed flagellated bodies. Figs. 1 and 2 show that the micronucleus and flagellum first divide, just as in trypanosomes, and next the macronucleus divides in turn, and a clear line appears in the length of the organism, indicating commencing division of the protoplasm of the body, as

shown in figs. 3 to 5 of the same line; while in fig. 6 the division of the body has just been completed, and in fig. 7 the micronuclei and flagella of a still adherent pair are dividing over again, thus showing how rapidly the multiplication was taking place in this culture, for such forms were not uncommonly seen in it.

In my earlier cultures the flagellated pairs were nearly always found in pairs only, although rarely three or four might be seen side by side. In the much more abundant development of flagellates in the acid culture medium, however, considerably larger masses, forming beautiful rosettes, with the flagella crossing each other in the centre, were seen in large numbers, and it is easy to understand how they may be formed by the rapid multiplication just described. Thus fig. 6 of Line IV shows a small group of flagellates which is remarkable for including nearly all the stages of division in a single clump, while fig. 9 shows the commencement of the formation of a rosette by the rapidly dividing flagellates pushing each other round to form a semi-circular mass, and in fig. 12 is shown a small, but complete, rosette, several of the forms in which are undergoing further subdivision. In this stage the contents of the eosin bodies frequently becomes protruded, as I have previously noted, and it accumulates round the flagella, helping to bind the forms together into the rosette shape. Next, the individual organisms elongate, and at the same time become narrower, and the rosette then commences to break up, in consequence of the increasing motility of the flagella, and some now separate from the mass in pairs or single forms as indicated in fig. 11, and in this manner the free swimming forms shown in Lines IV and V of the plate are produced. In fresh specimens these are very active, the single ones in particular threading their way rapidly among the red corpuscles, and on reaching an open space, dart about in such a manner as to leave no doubt in the mind of the observer that the object of this remarkable development and extraordinary increase in size is to endow the motionless human stage of the organism with the power of locomotion required in some period of its extra-corporeal existence.

The Nature of the Fully Developed Flagellate Form of the Organism.

When I first obtained the development of the flagellated stage, I thought them to be young trypanosomes which had not yet formed an undulating membrane. In support of this possibility, the recent observations of Novy and MacNeil (12) on the culture of trypanosomes of birds on blood agar are of great interest, for they obtained forms, separate and in rosettes, most closely resembling those shown in the plate accompanying the present paper,

both in the absence of all trace of undulating membrane and in the position of the micronucleus or blepharoplast at the anterior flagellated end of the organism, although in addition they obtained forms showing the development of the membrane by the passage of the blepharoplast back towards, and then past, the macronucleus, until it arrived near the posterior end of the organism, and a typical trypanosome resulted. When further experience of my culture failed to reveal any forms with a complete or even partial undulating membrane, the question arose whether it was not an organism distinct from the trypanosomes, although closely related to it, such as a hepatomonas in which no undulating membrane is present. In my last paper I left this an open question, while stating that nothing had yet been found which might not be an incompletely developed trypanosome; a view which has also been adopted by both Christophers (10) and Leishman (11). The more abundant and uniform development of flagellates in the acidified medium have enabled me to study closely innumerable apparently completely developed long free forms, in a stage in which they show extremely active movement in fresh specimens; but still no trace of an undulating membrane, or even a tendency for the micronucleus to pass away from the anterior end of the organism towards the macronucleus has ever been observed, although seen by Novy and MacNeil in their cultures of bird trypanosomes. I therefore conclude that the organism I have been able to develop belongs to the order Hepatomonas and not to the trypanosomes, and I propose to name it the *Hepatomonas of Kala-azar*. At the same time I prefer to limit the term kala-azar to the epidemic-spreading form of the disease as seen in Assam, and to retain the term "cachexial fever" for the less fatal sporadic affection, if only for the sake of avoiding the unnecessary cruelty of having to tell sufferers from the milder disease that they are suffering from the greatly dreaded kala-azar.

Degenerate Forms.

I have already pointed out that the absence of bacteria is necessary for the continued development of the flagellated stage of the organism, and that cocci especially are inimical to its growth. In one of my most active recent cultures staphylococci gained access to the tube on the seventh day of the culture during its repeated examination, and the degenerating changes resulting were readily followed. On the following day fresh specimens showed that all motion of the flagella had ceased, although on staining many of the organisms showed little or no change. Others, however, were granular and stained more lightly, while some were becoming shorter and more oval or

pear-shaped, and their flagella shorter, as in figs. 2 to 6 of Line VI, thus showing a tendency to reversion towards the undeveloped spleen stage of the parasite, only all stages of the degenerative process were present at the same time, and many of the shrunken badly staining forms were disintegrating. During this process the flagella were often shed, and with it the micronucleus came away, although a narrow non-staining space was still visible between the two, as shown in fig. 10, clearly proving an organic connection between the flagellum and the micronucleus or blepharoplast. Within three days all the rosettes of flagellates had broken up into granular masses and their identity completely lost. The degenerative changes in this hepatomonas are therefore very similar to those which have been described in the case of trypanosomes.

The Relationship of Leucocytes to the Parasites in Cultures.

Although it is doubtful as yet whether the Leishman-Donovan bodies can be found in the peripheral blood either free or in the red corpuscles, yet both Donovan (4) and Christophers (13) have found this stage of the parasite within leucocytes in the circulating blood during high fever, the latter having twice found a number of them, nearly all within polymorphonuclears, during a differential count of 500 leucocytes, which would mean an enormous number within the peripheral circulation at one time, and amply sufficient to infect a blood-sucking insect if such proved a suitable host. It is therefore of interest to determine if the parasites can develop in acid cultures within leucocytes. Figs. 7 and 8 illustrate conditions bearing on this point, the former representing a polymorphonuclear on the second day of the culture, which contains typical parasites, although they are somewhat less developed than those shown beneath it from the same slide; the latter shows another degenerating leucocyte from the same culture on the following day, in which some of the parasites are clearly much enlarged and developing typically, if somewhat more slowly, than those show outside the corpuscle, while others are degenerating and staining feebly. It appears then that development may proceed within leucocytes, while Christophers is also of opinion that it occurs within macrophages in cultures, so biting insects might be infected by the leucocytes containing the undeveloped parasites which have been found in the peripheral blood.

The way in which the polymorphonuclears especially take up the parasites in the peripheral blood is also of great interest in connection with the extreme decrease in these corpuscles, for I have shown that they are commonly decreased a tenth of the normal number, while in the latter stages of the disease, in children especially, they may fall to only from one-

twentieth to one-sixtieth, thus readily accounting for the frequency of terminal infection by such diseases as dysentery, cancrum oris, pneumonia and phthisis, owing to loss of phagocytic power, while I have also found the opsonic index reduced against the staphylococcus *pyogenes aureus*, which is frequently present in the spleen in cases of cancrum oris.

The Bearing of the Flagellation of the Parasite in Sterile Acid Medium on the Probable Mode of Infection.

The two factors which I have found most essential to a uniform development and very rapid multiplication of the flagellated forms are sterility and a slightly acid, or, at least, a neutral medium. I have also tried blood agar after Novy's method, only using human blood in its preparation, but failed to obtain either sub-cultures of already developed flagellates or of the spleen parasites, while only very scanty development was obtained when several drops of spleen blood, with very numerous parasites, were added to a previously acidified blood agar tube, and then only in the added blood as by the ordinary method. Now the only condition under which the Leishman-Donovan bodies would be likely to meet with a sterile acid medium on their escape from the human body would be in the stomach of some blood-sucking insect, of which the common bed bug, or possibly mosquitos, are the most likely hosts, for clinical reasons I have elsewhere pointed out, while I have found that after sucking blood it becomes acidified in gastro-ntestinal tract of bugs, and is also frequently sterile. I have not yet succeeded in inducing these insects to suck infected spleen blood placed in capsules of various kinds, but, on the other hand, I have mixed the contents of their stomachs after feeding on human blood (which was proved to be free from anything resembling any stage of the Hepatomonas of kala-azar) with about an equal quantity of spleen blood containing the parasites, and, after incubating in capillary tubes at 22° C., have been able to watch the development of the parasites day by day up to the flagellated stage under these conditions in those which remained sterile, but not when any bacteria were present. It is therefore clear that the conditions met with in the stomachs of bugs—and possibly also of mosquitos—are not inimical to the development of the parasite into the flagellated stage, provided the temperature conditions are suitable.

The more difficult question whether opportunities for infection of such insects occur sufficiently frequently to account for the incidence of the disease remains to be considered. In the first place, it is conceivable that bugs especially might become infected from skin lesions containing the parasites, for these may occur on parts of the body little exposed to the bites of

mosquitos, but, in my experience such skin affections are too rare to alone account for the frequency of infection. Further consideration will, I think, show that the difficulty in finding these minute parasites in the peripheral blood does not necessarily exclude the possibility of their occurring there in sufficient numbers to infect insects, especially during high fever, when they have been found in circulating leucocytes. In the first place, it has been shown, by Christophers especially, that the organisms multiply in the endothelial cells lining the blood-vessels of the internal organs, such as the spleen, liver, and bone-marrow, and when numerous, in films obtained by spleen puncture, they are frequently seen in groups in fragments of these cells, which during life must frequently rupture and set them free in the circulation, as is also proved by the same observer having found them in the blood of some of the large veins. It is further of interest to note that the endothelial cells of these very same organs are the principal sites of the deposits of malarial parasites in the internal organs, while I have also several times found Leishman bodies in the brain (where malarial parasites also occur), so that it is clear that they must frequently enter the circulating blood in considerable numbers. Secondly, the human stage of the parasite is so small that it would be scarcely easier to find in the blood by microscopical examination alone that typhoid bacilli in that disease, although the latter can be readily obtained by cultural methods.

The great difficulty of finding the human stage of the Hepatomonas of kala-azar in the blood, even if present in sufficient numbers to infect suitable insects, is well shown by Novy and MacNeil's (12) experience of searching for trypanosomes in birds; for while they only succeeded in detecting this large actively moving parasite by microscopical examination of thick blood films in 8 per cent., nevertheless they cultivated the parasite on their blood-agar medium in 50 per cent. of the same series. Moreover, even when they found them by their movement in thick fresh films, yet in the same birds they frequently failed to detect them in stained specimens. How much more difficult would it be to demonstrate the minute motionless Leishman bodies, which can be only seen in thin stained films, even if they were present in relatively large numbers in the peripheral blood?

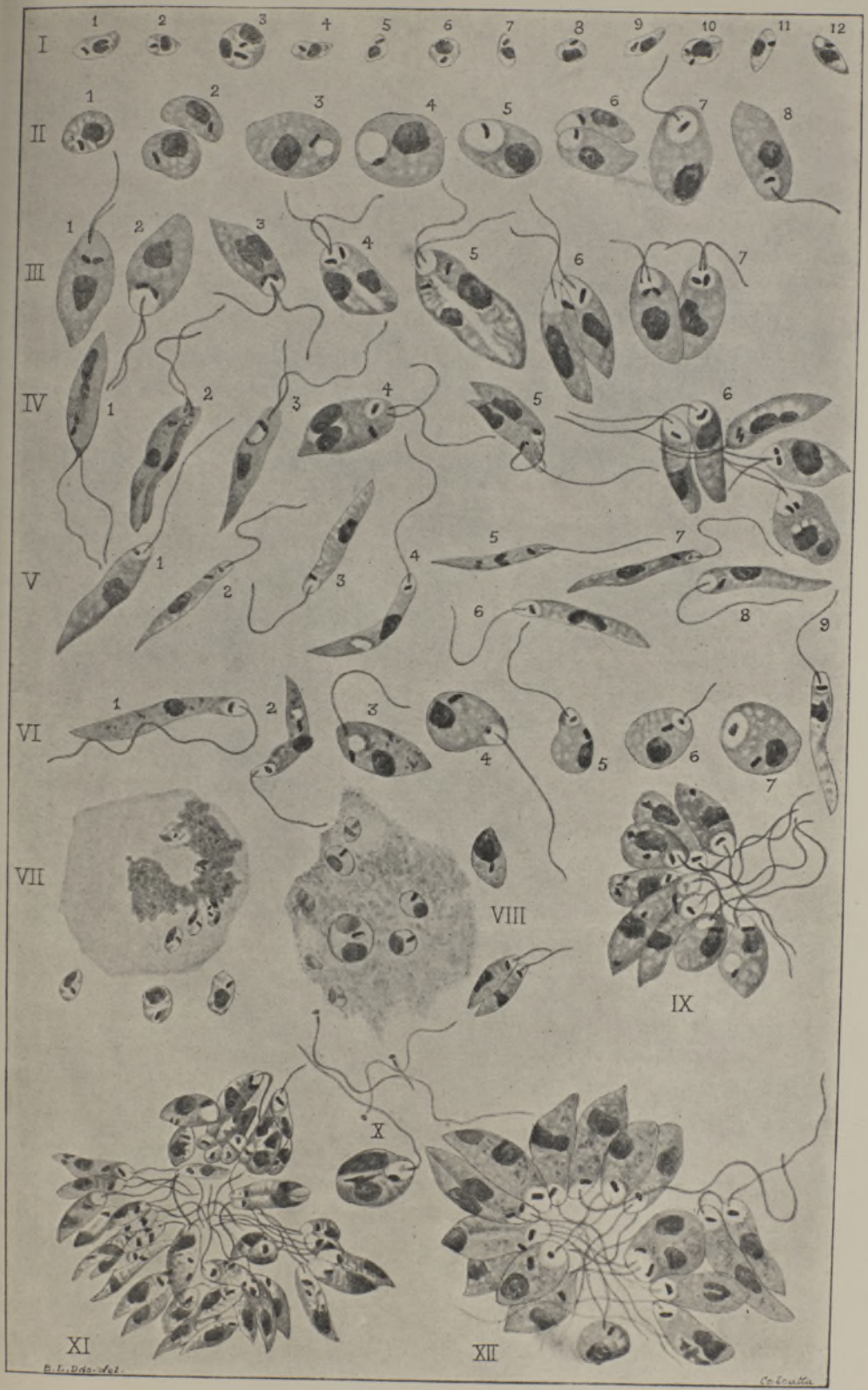
Thirdly, the extremely rapid multiplication of the flagellated forms in some of my recent cultures would appear to indicate that, in the presumably still more favourable natural conditions of the extra-corporeal stage of the parasite, a very small number of the human organisms would multiply to such an extent as to constitute a powerful infective agency.

The only reasonable alternative to the hypothesis just set forth is the suggestion of Manson, Christophers, and others, that the organism may escape

from the body by means of ulcers sometimes found in the intestines, the granulation tissue of which contains the parasites, and they may thus reach water. Apart from the great rarity of such infected intestinal lesions in my very extensive *post-mortem* experience of this disease in Assam and Calcutta, the fact that sterility is essential for the continued development of the flagellated stage of the organism appears to me to make this mode of infection an exceedingly improbable one. Moreover, I have been unable to obtain any development of the organism in even sterile water kept at the most favourable temperature, while even in sterile acidified water similar negative results have recently been obtained.

Relationship of the Optimum Temperature for the Development of the Flagellates to the Seasonal Incidence of Kala-Azar and Cachexial Fever.

If the conditions I have found necessary for the development of the flagellate stage of the Hepatomonas of kala-azar afford any indication of the natural conditions under which it occurs, then the striking fact that the relatively low temperature of about 22° C., or 72° F., is essential to the process, would indicate that infection is only likely to take place in India during the colder part of the year. Owing to the fever in this disease lasting for many months, or even several years, with long intervals of little or no rise of temperature, while cases not infrequently begin very insidiously, patients presenting themselves with marked, but often unsuspected, enlargement of the spleen, and a history of only a few days' fever; it appears probable that the incubation period may be a long one, and the onset very insidious and indefinite. Nevertheless a clear history can often be obtained, and an analysis of the notes of a number of cases showed five times as many in which the symptoms first commenced in the six months from November to April as in the remaining six hot months of the year, so that the cold weather months, together with the very commencement of the hot weather, to allow for the probable incubation period, show a very marked preponderance of the infection. Moreover, Dr. Dodds Price, of Assam, informs me, as a result of his unique experience of kala-azar, extending over 15 years, that every case he has seen in Europeans began in the cold season, and that among his hundreds of native cases he has noticed the same marked tendency for definite symptoms of the disease to first show themselves at that time of the year. The practical importance of this point in relation to the prevention of the disease is evident, while the close agreement of its seasonal incidence with the deductions from my experimental data is of considerable interest. It is also worthy of note that this disease is most prevalent in just those parts of India where the temperature conditions for



several months of the cold season most closely correspond with that which I have found to be most favourable to the development of the flagellated stage of the Hepatomonas of kala-azar, namely Assam, Bengal, and Madras. On the other hand, the disease is much rarer, or has not yet been proved to originate, in those parts of India where the winter season presents a greater degree of cold, and the more favourable spring and autumn are very short.

Much work will be necessary to test the truth or otherwise of the above hypothesis, but knowledge should mean power to prevent the most terrible of all tropical diseases in its combined very high mortality and slow death by inches, and as the most favourable cold weather working season is approaching, it appears to be advisable to put these observations on record for the benefit of other workers in this very important field of tropical medicine.

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DESCRIPTION OF PLATE.

Magnification of all the figures 1500 diameters.

FIG. I.—Undeveloped Leishman-Donovan bodies from spleen puncture film.

" II.—Early stages of development, from two days' culture in acidified citrated blood; 1 and 2, body and macronucleus enlarged; 3 and 4, first appearance of eosin body; 5 and 6, elongation and subdivision; 7 and 8, first appearance of flagellum.

" III.—Stages of division of the early flagellated forms.

" IV.—Double long swimming forms.

" V.—Fully developed long, free, active single cells.

" VI.—Degenerate forms.

" VII.—Undeveloped forms in a white corpuscle.

" VIII.—Early stages of development in a degenerating white corpuscle.

" IX.—Stage in the formation of rosette.

" X.—Separated flagella with micronuclei attached.

" XI.—Rosette breaking up into free forms.

" XII.—Small complete rosette.