Original Articles.

REPORT ON THE CULTIVATION OF PROTEOSOMA, LABBÉ, IN GREY MOSQUITOS.

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I.—Preliminary.

1. *Abstract of Former Researches.*—In a report to the Director-General, Indian Medical Service, dated 19th September 1897, I described some peculiar pigmented cells found by me in August 1897 in two dappled-winged mosquitos fed on blood containing crescents, and anticipated that they were a stage of the parasite of malaria in those insects. An abstract of the report was published in the *British Medical Journal* (1), to which were appended some remarks by Dr. Manson, Mr. Bland Sutton, and Dr. Thin on the original specimens, which they had seen. While Dr. Manson thought that the cells might be the extra-corporeal phase of the parasite of malaria, it was, on the other hand, considered possible for them to be either normal cells of the mosquito, or some parasite in it quite independent of malaria. In September 1897, however, the cells were seen again in two ordinary mosquitos fed on malarial blood; and the fresh light which these new observations threw on the subject enabled me to show (2) that they must be at least some kind of pathological growth in the insect; indeed, little doubt now remained in my mind that they actually were what I had from the first supposed them to be.

2. *Abstract of Recent Researches.*—I have lately been able to obtain conclusive evidence to this effect by having succeeded in producing these pigmented cells at will in grey mosquitos by feeding them on birds infected by proteosoma, Labbé—a parasite very similar and closely related to the hæmamœbæ of malaria in man. Numerous control experiments in which mosquitos of the same species were fed on healthy men and birds, or on men and birds with other gymnosporidia (to which order all these parasites belong), invariably yielded negative results. Further examination of the cells showed them to be the early stages of parasites, which infest the external wall of the insect's stomach, which in six or seven days reach a growth of no less than 60 μ or 70 μ, and which are finally extruded from the organ into the general body-cavity of the host. There is no doubt then that these parasites are a development in the mosquito of proteosoma in birds; and to judge from their structure and mode of growth, so far as yet observed, I take them to be *coccidia*.

II.—Results of Feeding various Insects on Blood containing various Species of Gymnosporidia.

1. *List of Former Results.*—Before describing recent experiments it is advisable to record briefly those made since I first took up the subject in May 1895, up to the end of February 1898. Each set of experiments or observations will be entered as a separate series.

Series 1.—From May 1895, out of a very large number of mosquitos of many species examined unfed, none contained pigmented cells.

Series 2.—From May 1895, out of a number of brindled and grey mosquitos fed on healthy persons, none contained pigmented cells.

Series 3.—From May 1895, out of a very large number of brindled mosquitos fed on patients with crescents, pigmented cells were not observed in any.

Series 4.—From 1896, out of a large number of grey mosquitos fed on patients with crescents, pigmented cells were not found in any.

Series 5.—In August and September 1897, out of four dappled-winged mosquitos fed on a case of crescents, pigmented cells were found in three.

Series 6.—In August and September 1897, out of a considerable number of small dappled-winged mosquitos fed on healthy persons, pigmented cells were not found in any.

Series 7.—In September 1897, one grey mosquito caught feeding on a case of mild tertian contained pigmented cells.

Series 8.—In January 1898, out of 30 horse-flies (Tabanus) and some lice fed on pigeons with halteridium, Labbé, none contained pigmented cells.

Series 9.—Early in February 1898, out of 34 brown brindled mosquitos fed on a case of triple quartan, none contained pigmented cells.

2. *Discovery of Pigmented Cells in Grey Mosquitos fed on Larks with Proteosoma.*—In February 1898, I was placed by the Government of India on special duty to prosecute these researches and was given the use of Surgeon-Lieutenant-Colonel D. D. Cunningham's Laboratory in Calcutta for the purpose. Steps were immediately taken to find again, if possible, the pigmented cells by feeding different species of mosquitos on blood containing different species of gymnosporidia. It was found that Calcutta abounded in the grey or barred-back species of mosquito, which is the filaria-bearing species, and with which I had already experimented in Series 2, 4 and 7 above. These had uniformly yielded negative results in Secunderabad when fed on blood containing crescents. Nevertheless, owing to difficulties in the way of obtaining other varieties, both of mosquitos and of malaria cases, numbers of this kind were again fed on a patient infected with crescents. Out of 41 of these searched exhaustively for pigmented cells, not one contained them (Series 10). An unrecorded number, say 50 more, examined unfed, or, after feeding on healthy persons, gave similar negative results.

As it was not the malarious season of the year, and as cases of hæmamœbiasis suitable for these experiments were not at all easy to

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procure, it was thought advisable to commence work on the so-called malaria parasites of birds. Accordingly, a crow (corvus splendens) and two tame pigeons infected with halteridium were obtained; and on the night of the 11th and 12th March these, with four short-toed larks (calandretta dukhunensis) and six sparrows (passer Indica), whose blood had not yet been carefully examined, were placed in their cages, all within the same mosquito netting, and a number of grey mosquitos of the species I had lately been experimenting with were released within the net. Next morning, numbers of these insects were found gorged with blood and were caught in test-tubes in which they were kept alive for two or three days.

On the 13th March I commenced to examine them. Out of fourteen of them, pigmented cells were at last found in one.

Believing as I did that these cells are derived from the gymnosporidia, I judged from this experiment that the grey mosquito which now contained them had fed itself on one of the birds which happened to be infected by a parasite capable of transference to the grey species of mosquito. As all the birds had been placed together in the same net, the question now was which of them had the mosquito fed herself upon. This could be easily ascertained. A number of mosquitos of the same species had meanwhile been fed separately on the crow and two pigeons with halteridium; but out of 34 of these examined not one contained pigmented cells. Hence I came to the conclusion that the mosquito with pigmented cells had not derived them from the crow and the two pigeons. The larks and sparrows remained.

The blood of these had not yet been carefully searched. I now found that three of the larks and one of the sparrows contained *proteosoma*, Labbé, and therefore thought it possible that the mosquito had been infected from one of these. Accordingly, on the night of the 17th and 18th March, a number of grey mosquitos were released on the three larks with proteosoma, and next morning it was found that nine of these had fed themselves.

On the morning of the 20th March, that is, from 48 to 60 hours after feeding, these nine insects were examined. Pigmented cells were found in no less than five of them. After the long continued negative experiments with this kind of mosquito (and indeed, I may say, after three years' doubtful attempts to cultivate these parasites) this result was almost conclusive. It indicated, as was surmised before, that when a certain species of mosquito is fed on blood containing a certain species of gymnosporidia, pigmented cells are developed. Hence it would follow, as an easy corollary, that the cells are a stage in the life-history of the gymnosporidium in the mosquito.

It was now necessary, however, to confirm and amplify this observation, and obtain formal proofs of the theorem, by repeating these experiments and by studying the pigmented cells themselves. Accordingly from the 18th March to the present date many differential experiments have been completed by feeding grey mosquitos as follows:—

(*a*) On larks, sparrows and a crow with proteosoma.
(*b*) On crows, pigeons and other birds with halteridium only.
(*c*) On a lark and a sparrow with immature proteosoma only.
(*d*) On a healthy sparrow.

Only the insects fed on group (*a*) have contained pigmented cells.

I will now give a list of these experiments, dividing them into series numbered in succession to those already recorded in paragraph 1 of this section. They will be found to illustrate in a striking manner the restricted association between the proteosoma in the birds and the pigmented cells in the grey mosquitos. Beginning from the commencement of my employment on special duty the series are as follows:—

3. *List of Recent Results. Series* 10.—Out of 41 grey mosquitos fed on a case of crescents (4th to 21st March), none contained pigmented cells.

Series 11.—Out of 15 dark-greenish dappled-winged mosquitos fed on a case of crescents, none contained pigmented cells. These insects were probably of the same genus, but not of the same species as those employed in Series 5, in which pigmented cells were found after feeding on blood containing crescents (4th to 21st March).

Series 12.—Out of 6 grey mosquitos fed on a case containing immature mild tertian parasites (night of 7th to 8th March) none contained pigmented cells (compare Series 7).

Series 13.—Out of 14 grey mosquitos fed on 1 crow and 2 pigeons with halteridium and 4 larks and 6 sparrows, some with proteosoma (night of 11th to 12th March) one contained pigmented cells.

Series 14.—Out of 34 grey mosquitos fed on 1 crow and 2 pigeons with halteridium (12th to 15th March), none contained pigmented cells.

Series 15.—Out of 17 grey mosquitos fed on 3 larks, all with proteosoma (17th to 19th March), 10 contained pigmented cells.

Series 16.—Out of 46 grey mosquitos fed on 2 larks and 1 sparrow, all with proteosoma (19th to 23rd March), 27 contained pigmented cells.

Series 17.—Out of 58 grey mosquitos fed on 1 crow with proteosoma caged together with 1 crow with halteridium (19th to 27th March), 40 contained pigmented cells.

Series 18.—Out of 3 grey mosquitos fed on 2 pigeons with halteridium (night of 20th to 21st March), none had pigmented cells.

Series 19.—Out of 18 grey mosquitos fed on 3 sparrows, two of which had proteosoma, while the third was healthy (24th to 26th March), 6 contained pigmented cells.

Series 20.—Out of 13 grey mosquitos fed on 2 sparrows, both with proteosoma (25th March to 1st April), 12 contained pigmented cells.

Series 21.—Out of 12 grey mosquitos fed on 2 larks, both with proteosoma (26th to 31st March), 7 contained pigmented cells.

Series 22.—Out of 10 grey mosquitos fed on 2 crows, both with halteridium only (night of 30th to 31st March), none contained pigmented cells.

Series 23.—Out of 25 grey mosquitos fed on a healthy sparrow (2nd to 7th April), none contained pigmented cells.

Series 24.—Out of 21 grey mosquitos fed on a sparrow with a moderate number of proteosoma (2nd to 3rd April), 21 contained pigmented cells.

Series 25.—Out of 26 grey mosquitos fed on a sparrow with numerous proteosoma (2nd to 11th April), 25 contained pigmented cells.

Series 26.—Out of 34 grey mosquitos fed on 2 sparrows (subjects of Series 24 and 25 above), both with proteosoma (7th to 16th April), 30 contained pigmented cells.

Series 27.—Out of 9 brindled mosquitos fed on 2 sparrows (subjects of Series 24, 25 and 26 above), both with proteosoma (7th to 9th April), none contained pigmented cells.

Series 28.—Out of 10 grey mosquitos fed on 1 lark and 1 sparrow, both with immature proteosoma (night of 9th to 10th April), none contained pigmented cells.

Series 29.—Out of 14 grey mosquitos fed on 1 lark with immature proteosoma (night of 10th to 11th April), none contained pigmented cells.

Series 30.—Out of 102 grey mosquitos fed on various crows with halteridium only (3rd April to 8th May), none contained pigmented cells. Of these 68 were fed in Calcutta and 34 in the Darjeeling district.

Series 31.—Out of 5 grey mosquitos fed on a maina (*acridotheres tristis*) with halteridium (night of 6th to 7th May), none contained pigmented cells.

Series 32.—Out of 5 grey mosquitos fed on a crested bunting (*melophus melanicterus*) with halteridium (night of 10th to 11th May), none contained pigmented cells.

Series 33.—Out of a few lice and horse-flies (Tabanus) fed on pigeons with halteridium (May), pigmented cells have not been found in any.

4. *Abstract of Recent Experiments with Grey Mosquitos.*—Thus we find:—

Out of 245 grey mosquitos fed on birds with proteosoma, 178, or 72 per cent., contained pigmented cells.

Out of 41 grey mosquitos fed on a man with crescents, five on a case of immature tertian parasites, 154 on birds with halteridium, 25 on a healthy sparrow, and 24 on birds with immature proteosoma, or a total of 249 insects altogether, not one contained a single pigmented cell.

5. *Some Particular Experiments.*—These experiments, then, are sufficiently decisive; it remains to discuss some of them more particularly. After the pigmented cells had been found in large numbers of insects fed on birds with proteosoma (Series 15, 16 and 17), it was thought time to commence some formal control experiments. Three fresh sparrows had been examined on the 23rd March after a day of fatiguing work with the oil-immersion lens, and no parasites were observed in them. A stock of mosquitos fed on them, however, showed pigmented cells in six out of eighteen insects (Series 19). The birds were examined again more carefully, when it was found that two out of the three did contain proteosoma after all. Indeed, when re-examined, one contained large numbers (namely, about 1 parasite to 50 corpuscles), the second contained a moderate number (about 1 parasite to 1,000 corpuscles), while in the third no parasites at all could be found. I now placed these three birds in separate cages, and put each cage within a separate mosquito-netting. A number of mosquitos caught in the larval stage in the same part of the same drain and hatched out in the same breeding bottle were now liberated at the same time (2nd April), partly on the first bird, partly on the second, and partly on the third. Next morning those which had bitten each bird were confined separately in test-tubes. On examination (Series 24, 25 and 26) it was found that out of twenty mosquitos fed at the time on the sparrow with many parasites, and out of the same number fed on the sparrow with a moderate number, all without exception, namely, 40, contained pigmented cells; while out of fifteen insects fed on the sparrow with no parasites, not one contained any pigmented cells. Moreover, the stomachs of ten insects of each of these series were preserved, and the number of pigmented cells in each series counted by means of a low power. The result was as follows:—

(*a*) Ten mosquitos fed on the sparrow with numerous proteosoma contained 1,009 pigmented cells, or an average of 101 each.

(*b*) Ten mosquitos fed on the sparrow with moderate proteosoma contained 292 pigmented cells, or an average of 29 each.

(*c*) Ten mosquitos fed on the sparrow with no proteosoma contained no pigmented cells.

I could not afford time to count with the oil-immersion lens. Had I done so, the number of cells found would doubtless have been larger. One of the mosquitos of series (*a*) contained no less than 445 of the cells. This experiment was repeated later with a few mosquitos and similar results were obtained.

Numerous experiments of the same kind were now carried on, by feeding mosquitos out of the same breeding bottle at the same time, (*a*) on birds with proteosoma, (*b*) on birds with halteridium. A large percentage of the former always possessed pigmented cells; none of the latter ever did so. For instance, on the night of the 12th to 13th April, a stock of mosquitos was fed partly on the two sparrows with numerous and moderate proteosoma put together, and partly on a young crow with halteridium. Out of twenty-three of the former twenty-two had pigmented cells; while out of sixteen of the latter none had them.

Crows gave interesting results. They are subject both to halteridium and to proteosoma; but I have found only one crow with the latter. In that one, however, the typical round sporulating form of proteosoma was observed. For some time the feeding experiments, for which I used various combinations of two or three crows at a time, had been giving contrary results; at one time positive, at another negative. On referring to my notes it was seen that the positive experiments occurred only when the crow with proteosoma had been present in the net (Series 6).

According to Manson's theory, infection of mosquitos should not take place unless flagellate bodies are present in the blood of the subject. Some experiments were designed to test this by means of feeding grey mosquitos on birds with immature proteosoma. No pigmented cells were found (Series 28 and 29). Further observations are, however, required in this connection.

Some of the experiments, namely, those on birds known to contain ripe proteosoma (Series 24, 25 and 26) yielded almost invariably positive results, namely, 76 mosquitos with pigmented cells out of 81 fed, or 94 per cent. Hence I think it quite possible that in future experiments made on carefully selected subjects, positive results may be obtained in 100 per cent. of the mosquitos.

An explanation may be required as to why insects were so often fed on *groups* of birds. The reasons are that wild birds become frightened and restless if caged all night alone, and that more mosquitos can be fed at once on several birds than on one, since, as I have actually experienced, small birds can be killed from loss of blood abstracted by too many mosquitos. Moreover, according to theory, the presence of flagellate bodies is necessary to infection of the second host; so that, especially when we have not had time to make a previous exhaustive study of the subjects' blood, the chances of causing infection are increased by using several birds at once instead of one. For more exact work, however, single feeding must be adopted.

6. *Deductions from the Feeding Experiments.*—There can be no question, then, that the pigmented cells are derived directly from the proteosoma. We can, however, already go further. The fact that similar cells were not found in control insects of the same species fed on blood containing other gymnosporidia will convince any one acquainted with parasitology that we are not dealing here with any mere physiological absorption of pigment by the stomach cells of the mosquito but with a vital phenomenon in the life-history of proteosoma, with a remarkable transformation by which the pigmented parasite in the blood of the bird become a pigmented parasite of some kind in the stomach tissues of the mosquito. It remains to add another mass of evidence to the same effect, deduced from the location, structure and growth of the pigmented cells in the mosquito; and to show how they undergo a development similar to that of filaria-embryos in the same host. Some details of the *technique* are, however, required as a preliminary.

III.—The Structure and Development of the Proteosoma-Coccidia in Grey Mosquitos.

1. *Technique.*—Mosquitos are obtained by catching the larvæ, or, better, the pupæ, in pots of water, drains, and puddles. These are placed in wide-mouthed glass vessels covered with muslin or stopped with a plug of cotton-wool, and will soon hatch out into the perfect insect.

The subject, whether a man or a bird in a cage, is put on a bed. The ordinary mosquito-netting (in good repair) used in tropical countries is suspended over the bed and is tucked under the mattress on which the subject has been placed. The jar of mosquitos is introduced under the netting and is opened, thus liberating as many of the insects as we wish. For birds a special netting may be devised.

Mosquitos gorged with blood are easily recognised at sight. They are caught by an assistant entering the net and placing a test-tube *slowly* over them as they sit on the gauze. A number of tubes containing a given stock of fed mosquitos is kept in the manner used in bacteriological laboratories for tube cultures; and the insects are killed by chloroform or tobacco-smoke.

Tubes containing mosquitos should be lightly plugged with cotton-wool; should have a few drops of water at the bottom for the inmates to drink, and lay their eggs in, and should be changed for fresh clean tubes every morning.

The females are fecundated shortly after feeding.

Grey mosquitos fed at night remain gorged and asleep for all the next day and night. Towards the end of the second day, however, the stomach begins to "clear," that is, the meal has been partly digested and partly evacuated, leaving the organ empty. The eggs are usually laid on the third day (with grey mosquitos), after which the insect generally dies if it has not been fed again in the meantime. In order to keep them alive for long periods they must be fed again every two days. In this manner I have kept them for twelve days; and, even after that, saw by the state of their ovaries that they could have lived longer. As with other animals, feeding is necessary to their existence. In order to refeed a stock, the test-tubes are simply opened under a mosquito-net containing a crow or sparrows, and the insects are caught again next morning as usual.

A mosquito is dissected for examination of the stomach in this manner. A needle, held in the left-hand, is passed through the thorax, the legs and wings are pulled off, and, if necessary, the scales of the body brushed off with a camel-hair brush. The tail is then lowered into a drop of water, salt solution or weak formalin placed on a glass slide; and with another needle held in the right-hand, the last two segments of the tail are partially separated and held down upon the slide. The left-hand is then moved in such a manner as to draw away the insect. If done skilfully, the alimentary and generative apparatus will remain on the slide attached to the last two segments of the tail. If necessary, as when the stomach is full, the last two segments may be entirely separated and the alimentary apparatus drawn out by the œsophageal end.

The alimentary canal consists of an œsophagus, a single sac, the stomach, and a short, straight (?) intestine leading to the anus and containing six heart-shaped glands near the rectum. Five long tubes, the malpighian tubes open into the stomach at its junction with the intestine.

The stomach is roughly about 2·5 mm. long and between ·5 mm. or 1·0 mm. broad when uncompressed by the cover-glass. Its ends terminate suddenly in the œsophagus and intestine respectively, the latter end being easily recognisable by the junction of the malpighian tubes (see Plate I, figure 23) Beginning at its outermost surface we have first the ramifications of the air vessels; next a series of parallel bands of annular and of longitudinal muscular fibres, crossing each other at right angles and leaving square interstices (which must not, of course, be mistaken for epithelium), and also some oblique bands; then a delicate homogeneous membrane which does not stain easily; then several layers of large cells which do stain easily and contain nucleus and nucleolus; and lastly, the lumen, containing blood, débris, water, bacteria, or nothing noticeable. I think that the outer coat is homogeneous, because I have observed no cellular structure in it; but this may nevertheless really exist. The cells beneath it have a diameter of about 30 μ when uncompressed and contain, besides the nucleus, numerous refractive granules of different sizes which are particularly large shortly after digestion. They often contain as well certain irregular but definite bodies lying close to the nucleus which may be of a parasitic nature; but I have never observed within them any of the granular débris from the cavity. Often, on the outer surface of the organ and among the branches of the air vessels, we find greenish, yellow or colourless globules of a high refraction, from 1 μ to 10 μ in diameter, free or enclosed in cells, and probably of an oily nature.

I have never observed gregarines in grey mosquitos—they seem to be parasites only of the brindled genus—but the intestine is often packed with hosts of monoflagellate organisms, about 6 μ in length, exclusive of the flagellum. These may be seen adhering to the wall of the gut, but breaking loose and swimming about actively on the addition of water. They have already been described by me as swarm-spores (3).

2. *Growth and Structure of the Coccidia.*—I can now proceed to a description of the development of the pigmented cells. It will be useful to suppose that a stock of grey mosquitos were fed on a sparrow with mature proteosoma on a *Saturday night;* and that at the same time control insects, whose larvæ were caught in the same drain and hatched out in the same breeding bottle, were fed on a healthy sparrow, on a man with crescents, and on a crow with halteridium, separately. Thus, insects dissected on Sunday, Monday, Tuesday, ... may be said to be dissected on the first, second, third, ... day, respectively, after feeding.

As the insects were released upon the subjects, say, at 6 P.M. on Saturday night, and were not caught again until, say, 6 A.M. on Sunday morning, and as they may have fed themselves at any time, or at several times during the night, it is clear that when they are killed the *period after feeding* will have a margin of uncertainty of twelve hours. An insect killed between 6 A.M. and 6 P.M. on Sunday, however, must have a period after feeding of between 0 and 24 hours; and one killed between 6 A.M. and 6 P.M. on Monday must have a period of between 24 and 48 hours; and so on. This method is wanting somewhat in precision, but unfortunately it is not easy to feed and capture a number of night-biting mosquitos all precisely at the same time, and it is exact enough for my present purpose.

First day, Sunday.—Taking a control insect fed on the healthy sparrow, we dissect it in the manner described above. The stomach full of blood, with the œsophagus, intestine, malpighian tubes and ovaries attached, lies before us on the glass slide. With the point of a needle we open the side of the organ, and the contained blood runs out freely into the water of dissection. On a cover-glass being now applied, its weight drives out almost all the rest of the blood through the rent we have made in the containing wall, and on holding the slide up to the light, we shall see the oval tissues of the stomach lying compressed, white and almost free from blood in the midst of the dark red-brown mass of its expressed contents. We can thus easily examine with the highest powers the stomach tissues or their contents, separately; or, if we wish, we can wash away the latter by irrigation with water or weak stains, and study the tissues alone, unstained or stained.

In the case of the mosquito fed on the healthy sparrow, we shall find that the *contents* consist of blood from which most of the serum has been abstracted, leaving the red and white corpuscles crowded together. What serum remains is densely coloured with exuded hæmoglobin, in the midst of which the white leucocytes, still living, can easily be distinguished. On examining the *tissues*, we find the cavity still containing traces of hæmoglobin-stained serum and perhaps a few leucocytes and red corpuscles; but, of course, neither the tissues nor the cavity show any sign of the characteristic pigment of proteosoma or halteridium.

Taking, however, an insect fed on blood containing gymnosporidia, whether hæmamœba, proteosoma, or halteridium, we shall find in the *contents*, besides the condition of the blood just described, not only traces of these parasites, but also entire parasites still enclosed in the corpuscles, or lying free in the yellow serum. In some of these the pigment is still oscillating. If filariæ or trypanosomes be present, some will be found living, others dead.

I will, for the present, make no attempt to describe further the condition of the parasites on this day, though the subject is one of the highest interest (Section IV, paragraph 1). I have thought it best to forego the study for the time, partly for this very reason, partly because the differential experiments noted above have necessitated the insects being reserved to a later stage at which the presence or absence of the pigmented cells becomes determinable with certainty, and partly because the enquiry was not absolutely necessary from a practical point of view, since an older development of the parasites had already been discovered. I will then pass at once to a study of the appearances of the second day.

Second day, Monday.—The blood in the cavity of the stomach is still further disintegrated. Early in the day we can indeed often distinguish a few red corpuscles and some rounded and dead leucocytes, but later on, as a rule, even these disappear, and the contents consist of a fairly homogeneous reddish-black mass of multitudes of granules with Brownian (?) movement. It should be noted, however, that the temperature of the air seems to have an appreciable effect on the period of these changes. Sometimes as early as the morning of this day, but more generally towards the afternoon, the stomach pours almost its entire contents into the intestine, which in its turn shortly evacuates them. From this point the alimentary canal becomes practically empty, but for a few traces of the meal, and the insect is ready to feed again. Meanwhile the eggs have been developing apace and the ovaries now appear as two thick yellow masses loosely attached to the alimentary apparatus and to the terminal segment.

Examining insects fed on blood infected with any of the pigmented gymnosporidia, we shall generally still find in the stomach *contents* some distinct traces of the parasites amongst the granular mass of the disintegrated blood. These traces consist either (*a*) of small clusters of pigment, (*b*) of scattered pigment granules, or (*c*) early during this day, of pigment contained in a yellowish, hyaline cell, the remains of the entire dead gymnosporidium. Pigment may also be seen in the dead leucocytes. Filariæ, if present, are dead.

If examining the *tissues* of the stomach in grey mosquitos fed on blood containing crescents, halteridium or *immature* proteosoma, after the contents have been evacuated either naturally or artificially, we shall, as a rule, find no trace at all of the parasites. Sometimes, however, we do find an occasional small *bolus* of pigment or some scattered granules of it; but these will be seen on careful examination to lie *invariably in the cavity*, in association with such small traces of the former meal of blood as have not succeeded in escaping from it—as indeed was to be expected. But the tissues themselves, whether the cells, the homogeneous outer coat, or the muscular coat, disclose no sign whatever of the parasites or of their typical pigment; they remain as entirely free from such as if the insect had been fed on healthy blood, or not fed at all; and it is quite clear that in insects fed as just described, the remains of the parasites which have escaped digestion have been voided amongst and as part of the normal excreta.

If, however, we now examine at the same stage a grey mosquito fed on blood containing mature proteosoma, a very different spectacle awaits us. On looking at the *tissues* of the stomach, we shall find the external muscular and homogeneous coats to be studded with small clusters of pigment identical in appearance with the characteristic pigment of proteosoma.

These constitute the youngest forms of the pigmented cells yet observed by me. It is necessary to examine them very particularly as regards (*a*) their position in the tissues, (*b*) their appearance and structure.

With a little skill, it is very easy to distinguish the various structures of which the mosquito's stomach is composed, especially when measures have been taken to prevent the cover-glass from unduly flattening out and crushing the tissues. On depressing the focus of the oil-immersion lens we shall come upon successively, first the ramifications of the air-tubes, with clusters of oil-globules; then upon the crossing bands of muscular fibre; then upon the outer homogeneous coat, in which no appearance of cellular structure is observed; then upon the layers of distinct cells; and lastly, upon the cavity, containing bacteria or traces of blood. Going further down we shall reach the other side of the stomach, with the cells, the homogeneous coat, the muscular fibres and the air vessels, all in a reverse order.

We shall find, then, no difficulty in locating the young pigmented cells. They lie invariably either in the external homogeneous coat or, still further outwards, actually between the strands of muscular fibre, which they separate in a manner with which we are familiar in the case of trichina embryos (Plate I, figure 5). I have never yet found them lying in or amongst the cells of the stomach; still less in the innermost layer of cells which we may perhaps consider as epithelial cells, or in the cavity of the stomach itself. This rule appears to be without exception. If the pigmented cells do not actually lie between the muscular fibres, they lie just beneath them (Plate III).

In appearance, the most elementary forms seem at first sight to consist of nothing but clusters of about 20 grains of pigment arranged within a small oval area, the longer diameter of which is about 8 μ, and the shorter diameter 6 μ or 7 μ. The pigment granules are however often seen to be arranged on the circumference of the oval; from which fact taken together with the observation that all similar clusters in the same insect appear to be of much the same size, we infer the fact that the pigment is contained within a cell. This is easily confirmed by further observations in which the containing cell is clearly visible.

In fact, taking insects killed on the morning of the second day, we shall generally find several varieties of these youngest pigmented cells. One variety presents the appearance just described of a mere cluster of pigment (Plate I, figure 1). Another variety consists of a delicate oval homogeneous plasma of a faint greenish colour, about 8 $\mu \times 6 \mu$ in size and containing about 20 granules of pigment variously arranged (Plate I, figure 2). In a third variety we have the cluster of pigment, within either a visible or an invisible plasma, and interspersed with some distinct *vacuoles*, and also some rather refractive *granules*, both of about 1 μ or 2 μ in diameter (Plate I, figure 3). In a fourth variety, somewhat larger in size, there are the pigment, the vacuoles and the granules, all enclosed within a very distinct oval outline (Plate I, figure 4).

These varieties have three things in common, their pigment, their oval shape and, approximately, their size. Often all the pigmented cells in one mosquito belong to the same variety and are nearly of the same size. Owing to the ambiguity in the time of feeding referred to above, it is difficult to pronounce as yet upon the question as to which of the forms is most elementary; but I think that the order of the varieties as just given is also the order of development. Thus the youngest cells would belong to varieties 1 and 2; later the vacuoles and granules appear; and lastly, we have the definitive outline. By degrees, towards the end of the second day, the earlier varieties disappear and the fourth form only is found, to remain as the type for some days (Plates II and III).

In some mosquitos of this day several varieties of the young cells are found together, and there is considerable variation in size. Such insects, I fancy, must have fed themselves on several occasions during the night.

The *pigment* consists of about 20 granules which have almost exactly the appearance of the pigment of proteosoma. The granules may perhaps be blacker and smaller and do not tend to form the little masses found in proteosoma, although they do tend to collect together in certain portions of the cell. Very often they are subject to a rapid oscillatory motion. Their arrangement is variable.

The *vacuoles*, when they appear, are very distinct and apparently full of clear fluid. I take the present opportunity for stating that throughout this paper I use the term "vacuole" only as a descriptive one. Their size often varies largely even in the same cell.

The *granules* appear to have more substance than the vacuoles, are usually all of much the same size, and look very like Labbé's "granules chromatoides" of halteridium.

The *staining* reactions, so far as observed, will be given in paragraph 3.

As we advance toward the evening of the second day, the cells become larger and larger; the vacuoles and granules become more numerous; the pigment tends to collect round one, or two, or more large vacuoles; and the outline is more and more marked. It is proper to notice, however, that there is a variety apparently descended from the early varieties without apparent plasma or outline, in which the outline is finer than in the ordinary forms, while the vacuoles and granules are much less visible. These seem to belong to the type of the "hyaline" forms of the third and fourth days.

At the end of the second day, that is, 48 hours after feeding, the cells may, I think, already reach a long diameter of 20 μ in a few cases.

Third day, Tuesday.—Grey mosquitos generally lay their eggs during this day on the surface of the water at the bottom of the test-tube, where they are to be found as small black floating "rafts" of eggs. After this the insects frequently die if they have not been fed again on the previous night.

In insects which have not been re-fed, we find stomach and intestine almost free from contents and the stomach cells very clear and hyaline. The eggs, if not laid, are fully matured.

On examining control insects fed on blood containing crescents and halteridium, no pigmented cells will be seen.

In those fed on blood with mature proteosoma, we shall find them in various numbers, and occupying exactly the same position as on the second day. They are, however, markedly larger and their outline is rounder; at the same time, it will be noted that they belong to one or the other of two varieties—they are either *vacuolated* or *hyaline* (Plates IV and V).

The vacuolated cells have generally a very marked and rather thick outline. They contain vacuoles and granules similar to those found in the larger cells of the previous day, but these are more numerous and more closely packed within the capsule. Some of the vacuoles are much larger than before, often reaching a diameter of 6 μ and even more; while the granules retain their former dimensions. Altogether these cells present a very substantial appearance and are at once distinguishable, even when out of focus, from the delicate tissues of the stomach.

The hyaline cells consist of a fine, sharp outline containing a perfectly clear plasma, in which however we can just discern some very faint vacuoles toward the centre of the cell.

In both varieties alike, the pigment tends to accumulate round and outside one or two large vacuoles, though scattered granules are generally present. The number of granules is, moreover, *less* than on the previous day, and their size is perhaps smaller.

In some mosquitos nearly all the cells belong either to the one or th- other variety, and that at the moment when the specimen is first made; in others the two varieties are found side by side. I have frequently observed that the hyaline cells have become vacuolated after ten or twenty minutes, but have never been so fortunate as actually to witness the change. This might lead one to infer that the latter are a *post-mortem* form of the former, were it not that both varieties may certainly be observed immediately after dissection and that perfect hyaline cells occur in insects which have been dead some hours. I can therefore offer no conjecture as to the nature of these differences in appearance between the two varieties.

As the third day advances, the pigment continues to decrease in amount; while, in some of the largest cells, a new element begins to appear, namely, a few bright refractive points scattered through the plasma. I take these to be minute globules of an oily nature comparable to those described by Leukart in coccidium oviforme; and shall in future denote them as such, although I am by no means confident in my opinion.

Meanwhile, the cells continue rapidly to grow in size until at the end of the day (72 hours) they may reach a major diameter which I put down at 30 μ in the largest cells. The average sizes for the day, however, lie between 16 μ and 25 μ, while cells, so small as even 10 μ may occasionally be seen. Indeed, I have often noticed great variations of size on this day.

Fourth day, Wednesday.—The cells have grown still more, and on the evening of this day (96 hours) may reach 40 μ in diameter. The averages lie between 25 μ and 35 μ, and I think that there is more uniformity of dimensions on this day than on the preceding one (Plate I, figures 13, 16; Plate VI).

If the insect examined be one which has survived to this day without refeeding, the cells may, I think, be stunted in growth and may scarcely exceed those of the third day in size.

If the mosquito has been fed again, say on the night of the second day (Monday night), the condition of the stomach and of the new crop of eggs (if present) will be that of the second day already described. If it has been fed again *on a bird infected with proteosoma*, as on the bird on which it was originally fed, the stomach will most probably be found to have on its external coat *a fresh generation* of *young pigmented cells*, in every way similar to those already found on the second day. On the margin of Plate VI, one such is figured lying next to some cells of an older generation. The new cells are evidently derived from the second feeding.

The two varieties of cells, vacuolated and hyaline, still exist. In both the pigment is arranged as before, and may still oscillate within a small area; but it is greatly reduced in amount, being sometimes reduced to only one or two granules lying close to a large vacuole and difficult to find. As the cells reach a major diameter of about 35 μ, namely, half the growth which they are destined to arrive at, the pigment disappears altogether, while the bright refractive points referred to as beginning to appear on the third day increase in number and perhaps in size. In the vacuolated variety the number of vacuoles and granules increases with the growth.

A noteworthy phenomenon manifests itself at about this stage. If the cover-glass has been placed so lightly on the stomach-tissues as not to crush them, and if a medium power be used, the pigmented cells will be seen protruded from all parts of the external wall of the stomach like warts on a finger (in Plate I, figure 23, this appearance is given, though the bodies there shown are of later growth than those now described); that is they protrude, not into the cavity of the stomach, but into the general body cavity, the coelom. This might perhaps have been inferred from the first position of the cells on the external coat of the organ.

From this point, then, the parasitic nature of these bodies becomes obvious, and we may, I think, venture to assume, at any rate provisionally, that they are coccidia. I will therefore now call them proteosoma-coccidia.

The identity of the cells of over 35 μ in diameter without pigment with those of under 35 μ with pigment is easily established by the similarity of structure in other respects. In many cases pigment may still exist in the coccidia which protrude from the tissues; and there is no possibility of mistake with regard to the continuity of development.

Fifth day, Thursday.—The coccidia continue to grow, and may reach a diameter of 50 μ at the end of this day (120 hours). Some of them not only protrude beyond the tissues, but may be seen to be almost distinct from them. The difference between vacuolated and hyaline cells is not so marked as before; and each coccidium now consists of a capsule containing a more or less granular and vacuolated substance studded with the numerous small bright points which I take to be minute oil globules. If the insect has been fed again on blood containing proteosoma, pigmented coccidia of a younger generation will generally be found interspersed among the older and non-pigmented ones (Plate VII).

Sixth day, Friday, and Later.—At the end of this day the coccidia arrive at a growth of 60 μ (144 hours), while some increase still further and may reach a size of nearly 70 μ. The largest cells seen by me measured, *when compressed by the cover-glass*, 73 μ by 62 μ. These coccidia are figured in Plate VIII after preservation in weak formalin.

Beyond this point I have observed no further growth, although I have examined coccidium-infected mosquitos kept alive to the twelfth day. Hence, I think, the dimensions here given may be considered as the maximum size reached by these bodies, at any rate in the living host. They are now easily visible by a magnifying glass, and are therefore nearly visible to the naked eye.

If mosquitos of this stage (or indeed earlier) be dissected in plain water, a curious phenomenon will be observed when microscope examination is commenced at once. The coccidia will be seen bursting in all directions, pouring out their contents slowly into the surrounding fluid, and leaving behind a collapsed and wrinkled capsule still attached to the stomach wall. This is probably due to the difference in specific gravity of the medium in which they now find themselves, and can be checked by dissection in weak formalin (10 per cent.), or perhaps in salt solution.

On examining a stomach immersed in formalin with a medium power of about 300 diameters, the coccidia will be seen everywhere, not only protruded but often extruded from the tissues, though still attached to them by some invisible adhesions. Each coccidium will have the appearance of a little, clear ball of glass containing numerous brilliant points, the oil-globules previously noted.

Some of them in addition possess a more refractive but ill-defined central core, which in a few cases I have observed to have the appearance of being divided into six or eight portions. These appearances, however, vanish on the imposition of the cover-glass, after which the coccidia, probably from being slightly flattened, present only a granular structure as shown in Plate IX. Seen by a still lower power, the stomach studded with coccidia has the appearance given in outline in Plate I, figure 23 ; where the mosquito has been re-fed on proteosoma-infected birds, younger generations of coccidia are seen embedded in the tissues or just emerging from them.

On applying the oil-immersion lens, the largest coccidia of the sixth and seventh day are observed to have a structure similar to that of the fifth day, namely, a more or less granular and vacuolated plasma studded with numerous brilliant points. In many of them, especially after preservation, there is now, however, a very marked contraction of the contents within the capsule (Plates VII and VIII, and Plate I, figures 16, 17 and 21) ; which, indeed, is to be seen in preserved specimens of an earlier stage of growth.

I have said that after this point I have witnessed no further growth of the coccidia, even in mosquitos kept alive to the twelfth day. Hence we may perhaps expect that they have become ripe for *sporulation.* No such thing has, however, been observed ; and we may therefore conjecture, unless I happened to have overlooked it, that it may occur either (*a*) in the living insect after twelve days, or (*b*) some time after the insect's death.

Further research is therefore required on this which now becomes the most important and interesting period of the life-history of the gymnosporidia, because on it depend our chances of future progress. To plunge into hypothesis for a moment, we may remark that since the mature coccidia find themselves in a closed cavity, namely, the body cavity of the host, there appears to be no means by which they can escape from that host during its life to undergo sporulation in external nature, as is the case with coccidium oviforme. It would appear then that sporulation should occur either within the living host, as with Eimeria, or within the dead host. The first would point to a completion of the life cycle by a direct infection of men and birds by the coccidium-spores in the mosquito ; the second to a more circuitous infection, perhaps by a second generation living free in water.

I will now close these descriptions, by giving a few notes on three peculiar forms of mature coccidia seen by me and figured in the plates.

(*To be continued*). pp. 448.

SOME POSSIBLE SOURCES OF INFECTION IN ENTERIC FEVER.

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(*Continued from page* 376.)

III—Beer.—Beer has not, as far as I know, ever been suggested as a source of infection of enteric fever. And yet apparently in this fermented beverage, the bacilli of enteric having once gained an entrance would grow and multiply exceedingly. Moreover, suspicion of it arises when it is remembered that the European soldier, who is the chief victim of enteric, consumes it daily as regularly as he does his ration of bread or meat, while the sepoy who so seldom contracts enteric presumably never tastes beer at all.

It may be said that as sepoys who never use fermented bread or beer sometimes contract enteric fever, these articles cannot be channels of infection. But it is not, of course, suggested that these are the sole channels of infection, only that they are possible sources of infection which are usually overlooked.

As in the case of bread, it may be well to examine first the making of beer or brewing, and then the articles used in such brewing.

Brewing is said to be the process of extracting a saccharine solution from malted grain, and converting this solution into a fermented sound alcoholic beverage called beer. It consists of two processes: first malting, and second brewing. Malting is the promotion of germination in barley or other grain by which some of its starch is converted into sugar. The grain is steeped for 48 hours and then turned and re-turned till root and stem begin to appear. It lasts about seven or eight days. The grain is then killed by drying. The second part of the process or brewing proper then begins.

The malt is crushed in a mill, then thoroughly mixed with hot water in a mashing machine, and then passed into a mash tun, a cylindrical vessel with false perforated bottom, through which a sweet liquor, the wort, drains. After three hours the wort is drawn off into a copper where the hops, or whatever substitute is used, are added, and the whole boiled for two hours to prevent acetous or putrefactive fermentation. When sufficiently boiled, the contents are run into the hop-back, a rectangular vessel with a perforated false bottom 8 or 9 inches from the true bottom. The hot wort passes through, leaving the spent hops, and thence into a cooler, where the wort is cooled to a temperature of about 100°F. Then it is supposed to be passed to a refrigerator, where it is cooled to about 58° F. Next, it passes to the fermenting wooden tuns where the yeast is added. During fermentation portion of the saccharine matter is converted into alcohol, and the temperature tends to rise considerably. To keep it down various methods have been suggested such as inverted metal cones filled with ice, or coils of copper piping through which cold water runs. When fermentation has proceeded far enough the liquor is allowed to settle, the beer becomes clear and bright and is filled into casks or vats, and, if stored for some months, is called "lager" beer. This beer is said to be slowly fermented at a low temperature.

Now, in the first part of the process of malting, the bacillus of enteric would find a capital environment for growth and multiplication, and also in the first part of the brewing process proper.

But after adding the hops the whole is boiled for two hours. This, according to our present