PRELIMINARY NOTE ON EXPERIMENTS IN CONNECTION WITH THE TRANSMISSION OF TICK FEVER.¹

By BREVET-LIEUTENANT-COLONEL W. B. LEISHMAN.

Royal Army Medical Corps.

The main facts relating to the transmission of tick fever are now well established, but uncertainty still remains as to the precise manner in which the virus of the disease, the Spirocheta duttoni, is conveyed by the bite of the tick and, also, as to the nature of the process by which the hereditary infection of successive generations of ticks is brought about. It has been known for some time that the young ticks born from an infected mother were themselves infected, but Möllers² has recently proved that the virus may also pass to a second generation of ticks without a fresh infection of the parent.

Again, for those who hold, as I do, that the pathogenic spirochetes are protozoal in their nature, the missing alternation of generation, which analogy would lead one to expect in the Arthropod host, has not yet been observed.

For these reasons, as well as on account of the results of some previous work with the S. duttoni,³ I endeavoured to obtain the material necessary for the investigations I had in view, and,

¹ The expenses of this investigation were in part met by a grant from the Royal Society.—W. B. L.
³ W. B. Leishman, Lancet, March 23rd, 1907, p. 806.
being fortunate in this respect, I have been working on the tick transmission of the disease during the last four months. The experiments are far from being concluded, but some of the results seem worthy of record in a preliminary communication, as they appear to me to throw some fresh light upon the subject.

Two years ago I received from Africa some of the ticks which transmit this disease, the Ornithodorus moubata, Murray, and endeavoured, by means of their bites, to infect susceptible animals, such as monkeys, rats and mice, but without success. A second consignment also failed. These ticks, however, furnished material for a study of their structure, and some of them laid eggs which hatched into young nymphs. A few ticks from these original consignments still remain and have furnished some control material in connection with the present investigations. I next communicated with the brother officer who had been good enough to send them to me, Captain Hallam Hardy, R.A.M.C., asking that the next batch should, if possible, be collected from native huts in which actual cases of the disease had occurred. This he most kindly did, and I have, within the last four months, received from him, from Nyassa-land, two batches of ticks collected under those conditions.

By the bites of these ticks, thirty-nine in one instance and eighty in the other, two monkeys became infected with tick fever, abundant spirochetes appearing in their blood after the usual incubation period of six days. The attack was fatal in each instance, the first monkey, bitten by the smaller number of ticks, succumbing during its second relapse at the end of the fourth week, while the other, presumably more heavily infected, did not survive its first attack, and died in ten days. Blood from these monkeys infected mice, and the strain of spirochete is now kept alive by passage through these animals.

A large number of ticks have been allowed to feed on these monkeys or on infected mice at times when spirochetes were abundantly present in the blood, and I had thus at my disposal the material necessary for the investigations. Many of these ticks, being fecundated adult females, oviposited two or three weeks after gorging with this infected blood and most of their eggs hatched out. They were kept in the dark at a constant temperature of 24° C. (Except where otherwise stated the ticks mentioned in this note were all kept at this temperature.)

When working with the earlier batches of ticks, those with which I failed to infect animals, the most careful examination of
the various organs and tissues failed to reveal the presence of a single spirochete; examination of many of the eggs laid by these ticks, and of larvae and nymphs hatched from their eggs, was also negative in this respect. In the case, however, of a few of the unfed young nymphs, examined after leaving the egg (the larval stage of this tick being passed within the egg-case), I encountered some curious collections of chromatin granules within the protoplasm of some of the cells lining a tubular structure, which I have since identified as a Malpighian tubule. These granules did not resemble any cell granulations with which I was familiar, and I strongly suspected them to be parasitic in nature, but, in the absence of any proof of the ticks from which they were born having been infected, I could not, of course, attribute to them any connection with the S. duttoni. I may, however, anticipate the observations recorded below by saying here that I now consider them to have such a connection.

Numerous observations upon the later batches of ticks which infected the monkeys also proved negative as regards the demonstration of spirochetes in any part of their body-fluids or tissues, an observation which it was difficult to reconcile with the generally assumed method of infection, namely, that unaltered spirochetes are inoculated into the animal, probably with the salivary secretion, when the tick bites.

The principal work upon this subject is that of Koch, who studied the disease in German East Africa in 1905. Koch found a very large number of the ticks infected in the districts in which he worked, as many as 60 per cent. in some instances, and appeared to have had no difficulty in detecting the presence of the spirochetes in the coelomic fluid. He also dissected ticks at various periods after they had been allowed to feed upon infected animals, and described the passage of the spirochetes through the thin walls of the intestinal sac into the ovary. In the ovary he found that the spirochetes penetrated into the immature ova and, following the ova after they were laid, and the embryos developing inside them, he still encountered clumps of recognisable spirochetes up to a point at which cell growth became so vigorous as to mask them. These observations appeared definitely to explain the mechanism of hereditary transmission of the disease in the tick, although it still left uncertainty as to the manner in which the spirochetes gain entrance to a fresh host when an infected tick bites.

The only confirmation of this part of Koch's work which I have encountered is the statement of Carter,¹ that he had observed spirochetes in the fluid withdrawn from the egg with a fine capillary pipette in the case of a small percentage of the eggs laid by an infected tick. On the other hand, other observers who have searched for the spirochetes under these conditions have failed to find them.

Without presuming to question the accuracy of these observations, I can only record my own failure to find a recognisable spirochete in any part of the body of a tick later than the tenth day after it had fed on heavily infected blood; and a similar failure in the case of eggs, whether dissected out of the ovary or laid under natural conditions. It is probable that differences in respect of some factor, such as that of the temperature or the climatic conditions, may explain these divergent results. At all events, it will, I think, be clear from the following experiments that there is a possibility of another method of hereditary transmission, as an alternative process, if not as the common one.

As to the methods employed in the experiments, the various batches of ticks fed on the same animal, and at the same time, have been kept isolated, as well as the eggs laid by such ticks and the young nymphs hatched from the eggs. Glass bottles with flat bottoms, filled with slips of clean filter paper, answered the purpose well, and a dish of water was kept in the incubator to prevent the air from becoming too dry. The latter detail I found to be necessary to allow of the hatching of sufficient numbers of eggs. After feeding, the ticks were usually placed in a vessel containing a little dry sand for a couple of days, as they like to bury themselves in this after they have gorged and the sand absorbs the Malpighian secretion and the coxal fluid voided at these times; later, they were removed to the bottles to allow of better observation as to their progress, the easy collection of the eggs, &c.

In examining the eggs, these have been washed in normal salt and then crushed on a clean slide, and a film made of the contents. Immature eggs, dissected out of the ovary, were treated in the same way. Unfed young nymphs and larvae were also crushed, as they are too minute for dissection.

Older ticks were pinned to blackened paraflin, and carefully dissected under the surface of salt solution, the isolation of the

various tissues and organs being greatly facilitated by the use of a binocular stereoscopic microscope. The various portions separated for examination were carefully washed and then spread out on slides if thin enough or, if too thick for this, were crushed with the flat surface of a surgical needle and films made from the crushed tissues. The various fluids or secretions were collected in capillary pipettes, and films were made from these in the usual way.

Examination of these was made either by the hanging drop method or after staining with my modification of Romanowsky's stain. In the latter case very deep chromatin staining was aimed at in all cases, and it was found necessary in most instances to wash the films after staining with a little 50 per cent. alcohol, as any trace of deposit added greatly to the difficulty of studying the extremely minute particles described below.

The observations so far made may now be briefly summarised as follows:—

(1) Spirochetes taken into the intestinal sac of the tick soon tend to lose their motility and, shortly after this, their characteristic appearance. I have never seen a motile spirochete in this situation, or, indeed, in any other, later than ten days after a tick has fed upon infected blood, and only rarely later than the third day.

(2) On the day after the tick has fed, the majority of the spirochetes are seen to have agglomerated into tangled masses, only those which are still isolated show any signs of motility. Shortly after this stage important structural alterations are manifest in stained specimens. These alterations appear to me to be of two kinds. First, the central core of chromatin substance shows evidence of segmentation into a number of definite rod-shaped blocks of chromatin, alternating with bands of pale blue substance. More rarely, the chromatin is broken up into deep-stained granules of oval or circular contour, which may be large enough to distend the spirochete at the point at which they occur. Secondly, in many instances there also appears a lateral swelling about the centre of the spirochete, which may attain a diameter of 1 to 2 microns, and is seen to contain one, or sometimes two, very deeply stained chromatin granules, apparently embedded in a pale, blue-staining matrix. A similar swelling is also sometimes seen at one pole of a parasite. The connection of the globular lateral swelling with the spirochete is at first sessile, but later becomes pedunculated, the body remaining adherent to the spirochete by a delicate pinkish thread.
The above morphological alterations are identical with those which I have observed in citrated blood containing either S. duttoni or S. recurrentis when such blood has been kept in the ice-chest for several days, and the forms which I found I demonstrated in June, 1907, at Edinburgh, at a meeting of the Pathological Society of Great Britain and Ireland. Similar changes have been reported in the case of these and of other spirochetes by various observers; thus, Breinl has noted them in spirochetes within the spleen, marrow and liver of infected animals, Carter has figured them in the spirochetes which he found in the eggs of infected ticks, and Dutton and Todd have also reported similar changes of duttoni in the intestinal sac and the Malpighian tubes of Ornithodorus. I may add that my present observations upon the changes of the spirochetes in the tick were made in ignorance of this last-mentioned work, so that they afford an independent confirmation of some of the observations of Dutton and Todd.

The changes which I had before noted in citrated blood were always suggestive to me as foreshadowing a possible change of form of the spirochetes in their alternative host, and the fact that similar changes do occur in this host lends additional support to the view that they are the commencement of an actual change of development, and not degenerative in their nature.

(3) Following upon these changes the segments of chromatin are apparently liberated from the body of the spirochete within which they were formed, as they are next found free in the intestinal sac and its diverticula. They were abundant in the case of a tick dissected on the second day after feeding. They exhibit no motility, and vary in appearance from a minute granule, approaching the limits of microscopic visibility, to small rods, either curved or straight, resembling, respectively, cholera or typhoid bacteria, but somewhat smaller (fig. 1). They appear to consist mostly of chromatin substance, but, in some cases, a small portion of blue-staining matter was noted in connection with the chromatin. On first encountering these bodies in the blood-mass of the intestinal sac, which occurred before I had been able to convince myself of their association with the spirochetes, I suspected them to be bacterial, but attempts to cultivate them on the usual media failed.

3 J. E. Dutton and J. L. Todd, Lancet, November 30th, 1907, p. 1523.
At the time when these free bodies are in evidence in the sac a number of very faintly staining spirochetes are to be seen, possibly those from which the chromatin has been extruded.

(4) The character and rapidity of the changes mentioned above appear to vary with the stage of attack in the animal upon which the tick was fed, and may possibly be further influenced by the time which had elapsed since the tick last fed, and by the temperature at which it had been kept.

(5) Soon after the occurrence of the above changes, as a rule on the third or fourth day after feeding, numbers of chromatin dots and rods, indistinguishable from those just described, are met with in the two following situations—First, in the cytoplasm of the cells lining the Malpighian tubules. In this situation the granules are frequently seen to be arranged in small clumps, varying from 2 to 5 microns in diameter, and such clumps, when liberated from the cells by crushing or laceration, are seen to possess a certain amount of coherency, and, in some instances, especially in the early days after the tick has fed, a pale blue-staining matrix can be made out by appropriate staining. Such clumps are, however, better seen and studied in the second situation. Second, in the ovarian tissue and in the oviducts. In this situation the clumps of granules are sometimes extremely abundant, especially if the animal upon which the tick was fed was heavily infected. The matrix is often seen in films made by crushing this tissue, but more often the clumps of granules are found to be surrounded by a clear zone or halo, which is not affected by the stain.

Prolonged study of these granules, in specimens taken from infected ticks which were dissected at various intervals after feeding, has left no doubt in my mind that they are derived from the spirochetes ingested by the tick. The numbers sometimes found have also given me the strong impression that an actual multiplication of the granules takes place in these two situations. They are so minute that I have not yet been able to follow the details of such a process, if it occurs, but it would appear probable that the clumps mentioned may be the result of a process of shizogony, and that such clumps or rosettes break up when mature and liberate the individual segments or granules, which are then free to multiply afresh and to form new rosettes. It is, however, also possible that some of the clumps may represent a simple aggregation of the segments derived from a single spirochete which had reached one of these situations in its spirillar form. It may also be mentioned that two types of these clumps occur; in one, the commoner, the
collection of chromatin bodies is composed of granules showing the same variation of shape as occurs in the contents of the intestinal sac; in the other form, the granules are all coccoid in shape, and there are none of the curved or straight chromatin rods. The latter type are more frequently found to be embedded in an unstained matrix.

Control material, in the shape of ticks which are known with certainty not to be infected, is, unfortunately, wanting, for the reason that the whole of my ticks have either come from an infected district or have been born from mothers which might have been infected. The fact that the grandchildren of infected ticks have been found infective, although their own parents had been bred in captivity and had never fed on infected blood, makes it difficult to be certain upon this point in the case of the ticks in my possession. Again, the fact that some of these ticks have failed to infect animals by their bites, to my mind does not in any way disprove the possible existence of the virus of the disease in some part of their bodies. The temperature at which a tick is kept is known to be a factor, and one which I have personally confirmed, and doubtless there are others of which we are still in ignorance. In other words, they may be infected but not necessarily infective.

At the same time, I have been able to convince myself that some of the ticks of the first batch, which have been nearly two years in captivity, and have never succeeded in infecting an animal by their bites, do not show any signs of the clumps of granules described above, either in the cells of their Malpighian tubules or in the tissues of the ovary. Again, as will be mentioned in a later section, the inoculation of tissues containing the granules has been followed by infection, when certain conditions were observed, while inoculation of the same tissues which had shown no evidence of the granules was negative.

As to the channel by which the granules reach the above situations, assuming my views as to their origin to be correct, there is little difficulty. The Malpighian tubules of this tick, two in number, are exceedingly long and delicate, they wind throughout the tissues of the tick in a very complex manner, and are, at many points of their course, in the most intimate apposition with the receptaculum and diverticula of the intestinal sac and with the ovary and oviducts. The distribution of the granules in the Malpighian cells is also irregular, large areas may show none, while, a little further along the tube, the cells may be densely
packed with them (fig. 2). This would suggest that the actual penetration of the intestinal wall by the granules or spirochetes takes place only where it is in contact with the Malpighian tubes or the ovary.

(6) Neither the chromatin granules nor recognisable spirochetes have been found elsewhere in the body of an infected tick, although systematic search has been made for them in the following situations: (a) the coelomic fluid; (b) the "fat-body"; (c) the salivary glands; (d) the "cephalic gland" of Christophers (this may possibly be identical with the structure recently named "Gené's organ" by Nuttall); (e) the coxal gland and coxal secretion; (f) the spermatheca; (g) the spermatophores; (h) the white secretion of the Malpighian tubules.

In the case of one infected tick, dissected on the fourth day after feeding, a number of short, curved, thread-like bodies were found in the ovary, associated with numbers of typical clumps of granules. The appearance and faint-staining properties of these threads suggested that they were either the remains of spirochetes from which the chromatin segments had been extruded, or, possibly, that they were young spirochetes derived from the granules. I have not encountered them again.

(7) A careful examination has been made of several hundreds of eggs which were laid by ticks known definitely to have been infected, or by such as had failed on some occasion to infect susceptible animals by their bites. In the great majority of the first class, those laid by certainly infected parents, I found clumps of the granules identical in every particular with those just described as occurring in the Malpighian cells and in the ovary. In a certain number, however, of the eggs of each batch laid by the same tick, none could be found. In the second class, those from parents which had not been proved to be infective, the clumps were also found, but in a very much smaller percentage of eggs. In some ticks of this class no granules could be found in any of the eggs examined.

Immature eggs have also been studied by careful dissection from the ovary under the microscope and, in many instances, the same clumps have been found in the egg-contents. In eggs of microscopic dimensions, 30 to 50 microns in diameter, one or more clumps have also been seen at times. In the case of one tick, infected by feeding four weeks before, which had been kept at a temperature of 37°C before dissection, the granular clumps were more numerous; one ovum, indeed, of which I have taken a photograph
(fig. 3), showed a dense mass of the granules inside the limiting membrane.

Eggs laid by certainly infected females have been studied from day to day after they had been laid and, in the great majority, the clumps have been found, often in considerable numbers (fig. 4). At first they are seen to be free in the yolk, but, after a few days further development of the egg, when cellular multiplication becomes manifest, the clumps were found to have entered, or to have been taken up by cells of mononuclear type, with abundant and deep-staining protoplasm (fig. 5). Some of these were demonstrated at a Laboratory Meeting of the Pathological Section of the Royal Society of Medicine, held at the Royal Army Medical College on November 3, 1908.1 From a study of more advanced eggs, of the embryo larva and of the young nymphs, I think that these cells are to be identified as the elementary constituents of the Malpighian tubules.

(8) Similar observations have been made, in great number, upon the larva and young nymphs hatched from these batches of eggs, and, in the majority, not in all, the same granular clumps have been found, invariably contained in the cytoplasm of the cells lining the short Malpighian tubules (fig. 6). Here there was no doubt as to the identity of the tubules, as the characteristic crystals of the Malpighian secretion were frequently observed within their lumen. I mentioned earlier the finding of certain curious aggregations of chromatin granules in similar tubes in dissections of young nymphs made about two years ago with the first batch of ticks; comparing these old specimens with those now in question the identity of the granules is manifest.

ANIMAL EXPERIMENTS.

(9) These are still in progress and it will suffice here to indicate their general character and the results of a few in which evidence bearing upon the mechanism of infection has been obtained.

The general idea of this part of the investigation has been to test the infectivity of the different fluids, tissues and organs of ticks at various stages of their development from the intra-ovarian egg upwards, controlling the results by a careful preliminary microscopical examination as to the presence or absence of spirochetes or the chromatin bodies which I consider to be derived from spirochetes. The tissues and organs have been isolated by

---

careful dissection under the microscope, washed thoroughly in several changes of sterile salt solution and then crushed in a watch­glass with a few drops of the salt solution. The resulting emulsion has then been inoculated hypodermically into white mice. The results of a few of these experiments may be briefly summarised.

A. In the following, the result of the inoculation was negative, the animal remaining free from spirochetosis.

1. Emulsion made from the crushed ova laid by a tick not known to have been infected. The tick and the ova had been kept at 24° C. Neither spirochetes nor granules were found.

2. Emulsion made from the Malpighian tubes of the same tick. Neither spirochetes nor granules found.

3. Emulsion made from the Malpighian tubes of ticks (two experiments) known to have been infected and kept at 24° C. In each instance granules were present but no spirochetes were found.

4. Emulsion made from the salivary glands of an infected tick which had been kept at 24° C. Neither granules nor spirochetes found.

5. Emulsion made from the salivary glands of an infected tick which had been kept at a temperature of 37° C. for two days before dissection. Neither spirochetes nor granules found.

B. In the following, the result of the inoculation was positive, the animals contracting tick fever and showing abundant spirochetes in their blood.

1. Emulsion made from the crushed walls of a diverticulum of the intestinal sac of an infected tick, together with a small amount of the contained products of digestion. The tick was one which had been fed upon a heavily infected animal a month before and had been kept for two days before dissection at a temperature of 37° C. No spirochetes were seen but a few granules were present.

2. Emulsion made from the Malpighian tubes of the same tick. No spirochetes but abundant clumps of the granules were found.

3. The white Malpighian secretion from the rectal dilatation of the same tick. Neither spirochetes nor granules were found, but the cells from the upper parts of the tube were filled with the chromatin granules.

4. Emulsion made from a small portion of the ovary of an infected tick which had been kept at 37° C. for seven days. Clumps of granules numerous, but no spirochetes.

The above experiments are naturally of interest and lend some support to my views as to the nature of the granules I have described. Needless to say, they are being repeated and extended.
I may conclude with a brief résumé of the observations, with the reservation that I am well aware of the many points awaiting further proof and confirmation, and of the absence of any ocular demonstration of the development of spirochetes out of the granules so frequently alluded to.

(1) After the ingestion by the tick of blood containing spirochetes these soon lose their motility in the intestinal sac and, eventually, their characteristic appearance. Morphological changes occur in them which result in the formation and liberation of small chromatin bodies, rod-shaped, coccoid, or curved in form.

(2) No recognisable spirochetes have been seen in any portion of a tick later than ten days after it had been fed on infected blood, nor have any been seen in the egg of a tick.

(3) The chromatin bodies traverse the walls of the intestinal sac and enter, or are taken up by the cells lining the Malpighian tubules; they are also to be found in quantities in the tissues of the ovary and the oviducts.

(4) Multiplication of the bodies within the tissues of the tick appears probable.

(5) Some of the bodies derived from the spirochetes penetrate into the immature eggs within the ovary and have been found in all the stages of the further development of the eggs, as well as in the earliest embryonal cells.

(6) In the embryo tick, the bodies are taken up by the cells lining the primitive Malpighian tubules and they have been found in the Malpighian tubules throughout all the subsequent stages of the tick's life.

(7) Inoculation of crushed tissue containing the chromatin bodies, but no spirochetes, has resulted in the infection of the inoculated animal when the tick from which they were obtained had been kept at a high temperature for some days before dissection.

From the above observations it appears to me possible that natural infection of tick-bite occurs, not, as usually supposed, by the inoculation of unaltered spirochetes from the salivary glands, but through the agency of these chromatin granules, which may either be regurgitated from the intestinal sac or passed in the Malpighian secretion which is voided by some ticks when gorging. In the latter case such granules might readily gain entrance into the new host through the wound inflicted by the tick's bite.
To illustrate paper on "Tick Fever," by Brevet-Lieutenant-Colonel Leishman, R.A.M.C.
DESCRIPTION OF PLATE.

The accompanying photo-micrographs were taken with Zeiss' apparatus from films stained by Leishman's method.

Fig. 1.—Film made from the contents of the intestinal sac of a tick, two days after it had fed on heavily infected blood. The chromatin bodies are seen lying among the black granules of digested blood, also a few pale-staining spirochetes. Magnification, 1,000 diameters.

Fig. 2.—Malpighian tubule of an infected tick, six days after feeding on infected blood, showing masses of the chromatin granules in the cells. Magnification, 1,000 diameters.

Fig. 3.—Ovum projecting from the edge of the ovary, from a tick infected four weeks previously, showing many clumps of the chromatin granules within its substance. Magnification, 2,000 diameters.

Fig. 4.—Clumps of granules in a film made from an egg just laid by an infected tick. Magnification, 1,000 diameters.

Fig. 5.—Embryonal cell from an egg in a more advanced stage of development, showing numerous clumps of granules, many within the cell. Magnification, 1,000 diameters.

Fig. 6.—Malpighian tubule of a young unfed nymph, showing a few masses of granules in the cells. Magnification, 1,000 diameters.