Cytological Studies on Heartwater.

II.—*Rickettsia ruminantium* in the tissues of Ticks capable of transmitting the disease (1).

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THE object of this study was to ascertain whether *Rickettsia ruminantium* previously reported (2) in the tissues of animals suffering from heartwater also occurred in ticks capable of transmitting the disease.

The methods of tick experimentation and the accurate system of records devised by Sir Arnold Theiler and Dr. du Toit were found to be most helpful. Through the foresight of Sir Arnold Theiler it was possible to experiment with larvae, all of which were the descendants of a single female *Amblyomma hebraeum*, and which might, therefore, be presumed to be readily comparable. All the ticks were kept in sterilized cotton-plugged test-tubes, and the tubes themselves were placed in glass jars, the bottoms of which were covered by thick layers of moistened sand. A large, well-ventilated room was devoted solely to tick experimentation. The ticks were protected from direct sunlight and any undue illumination. The history of the larvae and of the experiments performed with them and their descendants is summarized in Table 1, which has been extracted from the laboratory records. In this table, passing from left to right, information is given from the origin of the female which laid the eggs through the subsequent periods of feeding and moulting to the engorgement of adult females destined to give rise to the second generation.

TABLE 1.

History of ticks employed and record of experiments with them.

Series No.	Origin.	Date.	Eggs laid.	Larvae hatched.	Larvae fed on.	Date.	Larvae dropped off.	Larvae moulted.	Nymphae fed on.	Date.	Nymphae dropped off.	Nymphae moulted.
577	Female from cattle 638	27/1/24	2/2/24	10/3/24	Bull 424	13/9/24	18/9/24					
604	517				Calf 420	19/8/24	25/8/24	1/10/24				
605	517				Sheep 8046	19/8/24	26/8/24	11/10/24				
606	517				Sheep 8046	19/8/24	28,29,30/8/24	11/10/24				
607	517				Calf 928	27/8/24	1/9/24	13/10/24				
614	517				Bull 424	13/9/24	18/9/24	20/10/24				
635	614				Sheep 8049	22/10/24	29/10/24	10/12/24				
636	614								Sheep 8049	3/11/24	7/11/24	10/12/24
637	607								Goat 9651	3/11/24	13/11/24	10/12/24
638	614								Goat 9665	3/11/24	13/11/24	12/12/24
640	614								Goats 8235, 8417	22/11/24		20/12/24
647	517				Cattle 928		17/12/24					

Experiments.

Five batches of larvae were taken from series 517 and were numbered 604, 605, 606, 607, and 614 respectively. The first three (604, 605, and 606) were fed on normal animals, as indicated, and the remaining two (607 and 614) were fed on animals suffering from heartwater. That the virus was actually in the blood at the time of feeding was proved by inoculation of the blood into susceptible animals, which, after the usual period of incubation, contracted typical attacks of the disease.

The engorged larvae of the infected series (607 and 614) were allowed to moult, and certain nymphae, which developed from them and were renumbered 636, 637, 638, and 640, were again fed upon animals, some of which prove to be susceptible, since they contracted heartwater, as will be related subsequently (see page 190). It was decided that statements as to the presence or absence of *Rickettsia* could only be based upon very thorough study of the ticks. For this reason, although comparatively few specimens were examined, it is probable that the results were more valuable than if many hundreds had been superficially observed. The histological examinations made are listed in Table 2. In the first column, the number of the series is given; in the second, the time the examinations were made after the ticks were fed as larvae (the days when Rickettsia were observed being underlined); and in the third, the total number of specimens studied containing Rickettsia, marked +, and without them, marked −.

Table 2.

Examinations.

Series.	Data.	*Rickettsia ruminantium.*
Original 517....	Unfed larvae 189 days after hatching.....	About 50−.
Control 604.....	Number of days after commencing to feed as larvae: 51, 53, 57–59, 61–64, 75–79, 84, 85, 96, 105, 112	0+ 60−
Control 605.....	Number of days after commencing to feed as larvae: 75, 104, 106, 111	0+ 35−
Control 606.....	Number of days after commencing to feed as larvae: 67, 75, 104, 111	0+ 47−
Infective 607....	Number of days after commencing to feed as larvae: 30, 58–60, 61, 62, 64, 67, 68, 70, 71, 81, 84, 89, 101	35+ 59−
Infective 614....	Number of days after commencing to feed as larvae: 13, 14, 16, 20 22, 23, 27, 28–34, 40, 41, 45, 46, 47, 50, 54, 55, 57, 68	20+ 102−
Control 635..... Originally 517	Number of days after commencing to feed as larvae: 47....	0+ 1−
Infective 636.... Originally 614	Number of days after commencing to feed as larvae: 75, 82, 96 (5 nymphae and 4 adults)	4+ 5−
Infective 637.... Originally 607	Number of days after commencing to feed as larvae: 96 (nymphae)	1+ 2−
Infective 638.... Originally 614	Number of days after commencing to feed as larvae: 96 (2 nymphae and 2 adults)	3+ 1−
Control 647..... Originally 517	Number of days after ceasing to feed as larvae: 5.....	0+ 2−

As a routine procedure and in the majority of cases the ticks were studied in sections, but this method was often supplemented by smears and by teasing out the living cells in sterile salt solution and observing them by oblique illumination and by direct illumination

unstained and with the aid of various vital dyes. Special treatment was naturally required in each stage of the life-cycle.

Unfed larvae soon after they hatched from the eggs (series 517) were of such small size that large numbers (twenty-five or more) were fixed (in Regaud's fluid) at one time. They were allowed to settle in a fairly compact mass in the paraffin before solidifying. The clumps of larvae thus formed cut with difficulty, but a few good sections were often obtained. In making smears, ten or more larvae were applied successively to a single slide. Care was taken to smear them roughly parallel so that each specimen could be followed from end to end with the aid of a mechanical stage.

Engorged larvae were also found exceedingly difficult to properly fix and section on account of the large amount of blood contained in their intestines, their very resistant integument, and their relatively small size. Accordingly, chief reliance was placed in smears and in the examination of living tissues. The smears were made, as in the case of the unfed larvae, with sterile instrument upon clean slides without the addition of any saline solution. By this method drying was almost instantaneous. Before staining, the smears were fixed in absolute alcohol, which gave better results than methyl alcohol.

The nymphae, being slightly larger and less brittle, were cut into sections comparatively easily. In order to prevent the chitin from hardening, the time required for dehydration and for embedding was reduced to a minimum. The penetration of the fixative was facilitated by the removal of the legs and by making a small incision in the integument. The sections were cut at a thickness of 4μ or 5μ, but even with the greatest care, a complete series of sections was never obtained. At first Zenker's fluid was employed as a preservative, but this was later abandoned on account of a crystalline deposit produced for the most part within the intestinal lumen. This deposit was not removed by the action of Lugol's iodine solution, followed by a 5 per cent. solution of sodium hyposulphite, but it was dissolved by allowing the sections to remain for a few minutes in a 1 per cent. solution of potassium permanganate, followed by rinsing them in 5 per cent. oxalic acid. This treatment, however, partially destroyed the contrast coloration with Giemsa's stain. For this reason Regaud's mixture was used, consisting of 4 parts of a 3 per cent. solution of potassium bichromate and 1 part of commercial formalin (3). The intensity of the subsequent coloration by Giemsa's stain was increased by the mordanting action of the formalin, so that it was necessary to render the staining fluid feebly alkaline and to add methyl alcohol as advised by Wolbach (4). Other fixatives and stains were employed for special purposes (see page 188), and the tissues were examined in smears and in the living state.

In the case of *adults* it was necessary to remove all traces of the chitinous exoskeleton in order to secure good sections. It was found that this was easily accomplished after a few hours' preliminary fixation in formalin or Regaud's fluid, which made the tissue firmer and greatly facilitated the stripping off of the chitin. Slightly better results were obtained when the ticks were partially dehydrated in the alcohols before commencing to remove the chitin, because this treatment made the viscera more cohesive. Another device was used with engorged nymphs, as well as with adults. This consisted of squeezing out the viscera through a small incision in the exoskeleton. A small

pair of artery clamps proved much more helpful than ordinary forceps, because with them the pressure could be regulated and maintained. After a little practice and with the use of a clean wet needle, the viscera could, in most instances, be induced to form a single drop, which kept its shape when it fell into the fixative, and was consequently very easily imbedded and sectioned. For this purpose Regaud's fluid gave poor results as compared with Zenker's fluid, Giemsa's sublimate, and sublimate acetic, because in it the tissue tended to disperse instead of retaining its spherical shape.

Three kinds of Micro-organisms.

From the outset a relatively large, Gram-negative, very pleomorphic, typically intracellular, bacterium-like micro-organism was invariably found to be present within the living epithelial cells of the Malpighian tubules of all ticks (larvae, nymphae, and adults), both infective and non-infective. This was the same micro-organism as that previously reported in the eggs of the specimens of *Amblyomma hebraeum* sent by Sir Arnold Theiler to the Rockefeller Institute (5). Since it was shown that it occurs likewise in unfed larvae (series 517) which had been reared in sterile test-tubes and had taken no food, it was concluded that it constitutes an hereditary infestation (6).

Rickettsia ruminantium, on the other hand, was only observed in infective ticks of the series 607 and 614. Because the life-history of both micro-organisms is but imperfectly known, there is always the possibility that in some stages they may be confused. It was found that this is particularly to be guarded against in the study of the multiplicative phases in smears when the two micro-organisms are nearly of the same size and their relations to the cells of the tick have been lost. They have, therefore, been contrasted in fig. 7. The large pleomorphic micro-organisms are seen in the cells of the Malpighian tubule to the left and small clumps of the much more minute *Rickettsia ruminantium* are represented in contiguous intestinal ephithelial cells on the right. The first are coloured red, and the second blue. Other criteria of distinction are listed in Table 3.

Table 3.

Comparison of the Pleomorphic micro-organism and Rickettsia ruminantium.

	Pleomorphic Micro-organism.	*Rickettsia ruminantium.*
Morphology.....	Rods and filaments 0·5–1·5 by 1–5 μ very pleomorphic	Cocci and diplococci pairs 0·25–0·3 by 0·5–0·8 μ very regular.
Staining reactions	Gram negative, usually red with Giemsa; after formalin fixation, stains lightly by Goodpasture's fuchsin method (7); fragile, not easily fixed; stains heterogeneously, revealing chromophilic areas	Gram negative, usually blue with Giemsa; after formalin fixation, stains electively and intensely by Goodpasture's fuchsin method; not fragile, very easily fixed; stains homogeneously, revealing no internal structure.
Position.....	Intracellular in egg-cells and Malpighian tubules	Intracellular and sometimes extracellular in alimentary tract, not in egg-cells or Malpighian tubules.
Occurrence.....	In all larvae, nymphae, and adults infective and non-infective, inherited through the eggs	Only in infective specimens and not inherited through the eggs.

Specimens of *Amblyomma hebraeum* of unknown history were found to be occasionally infested with a third type of micro-organism (5)—a Gram-positive bacillus, approximately 0.25μ by 1μ, parasitic in the cells of the salivary glands; but this micro-organism was not observed in the carefully protected ticks under experimentation. It is only mentioned here so that subsequent investigators may at once eliminate it in any consideration of the etiology of heartwater.

Distinctive properties of *Rickettsia ruminantium* as observed in Infective Ticks.

Attempts to examine *Rickettsia ruminantium* in the living state, within intestinal epithelial cells teased out in sterile saline solution and in the contents of the gut, yielded only indecisive results owing to uncertainty in identification. The main difficulty was to distinguish between the Rickettsia and some granules from the salivary glands of about the same size, which tended also to occur in similar spherical clumps. But the differentiation was clearly made upon the addition of a little of the relatively pure medicinal methylene blue of Meister, Lucius, and Bruning (pre-war product). In a concentration of about 1:20,000 this dye coloured both the granules and the Rickettsia, the latter rather more intensely. The Rickettsia were less refractile and did not possess the definite contours of the granules. They were not motile. Each blue-coloured Rickettsia seemed to be surrounded by a very thin layer of unstained substance, which created the illusory impression that the Rickettsia were less closely packed together with the granules. When Brilliant cresyl blue (Grübler) was applied in place of methylene blue, the Rickettsia became stained light purple, but were not so clearly seen. The Rickettsia were also coloured by neutraal red (Leopold Casella & Co.), but it was more difficult to detect them owing to the strong affinity of many other granules for this dye.

The examination of living cells and of the contents of the gut by dark field illumination, even under the most favourable optical conditions, proved unprofitable because myriads of mitochondria of approximately the same size and shape as the Rickettsia were revealed so clearly that any attempt at differentiation between the two would have been both arbitrary and futile. This confusing element could perhaps have been predicted on the basis of observations recorded in the literature relative to the mitochondria in *Ixodes reduvius* (8), *Argas mineatus* (9), and *Amblyomma americana* (5). The distinction between mitochondria and Rickettsia was made beyond peradventure in fixed tissues, as will be related in the following paragraphs.

Much information was gained by the study of air-dried smears fixed in absolute alcohol and coloured by Giemsa's method. But in the examination of the smears it was necessary again to carefully distinguish between the Rickettsia and these granules of the salivary glands. Like the Rickettsia, these granules often occurred in spherical clumps and were of very minute and fairly uniform size, but they always stained (by Giemsa) a light brick-red colour and differed from the Rickettsia in the finer points of their morphology. By contrast the clumps of Rickettsia were usually coloured blue or light purple (fig. 3), and in places where the individual micro-organisms had been separated it was observed that many of the Rickettsia were composed of two materials, one coloured red and the

other very pale blue, as illustrated in fig. 1. The red staining material was present in two minute spherules embedded in the blue staining substance which intervened between them. The ends of the pairs, composed of the red staining spherules, were uniformly rounded. The intervening blue staining substance frequently possessed a greater diameter than the spherules. The distance between the spherules was greater than that generally seen in the case of pairs of diplococci. In some cases this distance varied somewhat, suggesting degrees of separation after transverse division. These properties called at once to mind the similar appearance in smears of the Rickettsia of typhus and of Rocky Mountain spotted fever and of *Rickettsia nipponica* as cultivated by Sellards (10). But a very exhaustive search failed to reveal any filamentous forms in the case of *Rickettsia ruminantium*.

It was, however, in sections that *Rickettsia ruminantium* was most profitably studied and its relation to the intestinal epithelial cells determined. Its most characteristic feature, already alluded to, was its habit of occurring in densely packed spherical clumps, precisely as in the endothelial cells of animals suffering from heart-water. This clumping is well illustrated in figs. 2 to 7, which should be compared with figs. 1 to 9 in the first part of this report dealing with the same Rickettsia in mammalian tissues (2). The clumps varied greatly in size, and several of them were often found within the cytoplasm of a single cell (figs. 5 to 7), just as in mammals. The tendency noted in mammals for the masses of Rickettsia to be surrounded by a kind of halo of clear cytoplasm, was less marked in the ticks, probably because the cytoplasm of the intestinal epithelial cells was very fluid and contained comparatively little stainable ground substance, or perhaps on account of the absence of intracellular digestion of the Rickettsia in them. In some cases in the ticks it was seen that these clumps were distinctly heterogeneous in character, being composed of the Rickettsia themselves, and of a matrix which differed in staining properties from the ground substance of the cells. This matrix was detected in specimens of series 607 fixed in both Regaud's fluid and 10 per cent. formalin and coloured by Giemsa's stain, which was only slightly differentiated. It is illustrated in light blue in fig. 4 *d* and *e*. Its occurrence, however, was not constant even in neighbouring clumps of Rickettsia in the same section, which were presumably coloured in exactly the same way. The matrix seemed to be most frequently met with in the case of masses of Rickettsia which were not very tightly packed together. Experimental evidence that the individual Rickettsia composing a clump were very cohesive was obtained in the examination of living tissues and of smears in which the clumps often remained intact (as represented in fig. 3), although they had been subjected to considerable mechanical traction. The clumps increased progressively in size and in number during the first few days after their first appearance, and, in the older nymphae, the Rickettsia were observed to escape into the lumen of the intestine. In this new location also they usually retained their clump-like association (in spite of the fact that they were suspended in a more fluid medium), but they were occasionally scattered about singly and in small irregular groups.

When closely crowded together the Rickettsia had the appearance of minute cocci, about 0.2μ to 0.3μ in diameter, as illustrated in the

photomicrographs, figs. 8 to 13. When, however, the plane of section passed barely through the edge of a mass of Rickettsia, the optical effect of superposition was avoided, and it was observed that some of them were bacillary in shape and others diplococcal. The bacilli measured about 0.2μ to 0.3μ by 0.4μ to 0.5μ, as seen after fixation in Regaud's fluid and coloration by Giemsa's stain. The pairs of diplococci were approximately 0.2μ by 0.8μ. It is important to note that the coccal forms were sometimes swollen by the action of formalin, as indicated in fig. 4 *f* and *g*.

Certain atypical bodies possibly, but not probably, related to the Rickettsia made their appearance in sections of ticks of series 607, examined as nymphae, seventy-eight days after they commenced to feed on an infected animal. They were larger than most Rickettsia, of bacillary or coccoid shape, sometimes disposed in pairs, and usually embedded in the substance of clumps of true Rickettsia. After Regaud's fixation they were coloured dark red by Giemsa's stain, instead of some shade of blue or purple. These atypical bodies are illustrated in fig. 4 *a*, *b*, and *c*, and in the photomicrograph, fig. 10. They were not seen in older nymphae which had become engorged with blood by feeding on animals to which they transmitted heartwater, although the regular forms of Rickettsia were abundant in the living epithelial cells of the intestine and free in the lumen.

The duality in staining property, observed in the smears, was not clearly seen in sections—an experience also reported by Hertig and Wolbach (11) in their study of Rickettsia in arthropods. In sections the Rickettsia of heartwater were usually stained only one colour, with the possible exception of the questionable atypical forms already mentioned. The reactions of the Rickettsia were, as was to be expected, more dependent upon the fixative (and its mordanting action) than upon the stain. For example, with Giemsa's stain they were coloured clear blue after Zenker's fluid, with or without acetic acid; bluish-purple after Regaud's fluid, formalin, or Carnoy's fluid; pale green after Flemming's fluid; and light pink after the picrosulphuric mixture of Mayer (12). The Rickettsia were Gram-negative and stained well by Goodpasture's fuchsin method and by Unna's alkaline methylene blue after appropriate preservations. In stained preparations their outlines were somewhat less distinct than most bacteria, a phenomenon which Da Rocha-Lima (13) noted in his description of the type species, *Rickettsia prowazeki*.

As observed in sections, the intestinal epithelial cells containing the Rickettsia showed no recognizable signs of injury, as was also the case with the Rickettsia-laden endothelial cells of mammals suffering from heartwater. This is an important point of similarity with the Rickettsia of Rocky Mountain spotted fever, as seen in the tissues of the tick *Dermacentor venustus*, and with many other Rickettsia, which typically establish relations with their arachnid hosts approaching true symbiosis.

In describing these Rickettsia in mammalian tissues, it was shown that they are true micro-organisms which need not be confused with mitochondria, or with any other normal cellular component, or with any product of cellular degeneration, or of injury. Much of the evidence then advanced is applicable also to these Rickettsia in ticks. It will suffice to recall their characteristic morphology and staining properties, their presence in infective ticks, and their absence in controls.

RELATION OF *Rickettsia ruminantium* TO INFECTIVITY IN TICKS.

Rickettsia ruminantium was observed in the two strains of ticks (i.e. 607 and 614) which had fed upon cases of heartwater and was uniformly absent in the three series of controls (i.e. 604, 605, and 606). But in series 614 it was detected considerably earlier in one larva which had fed upon a case of heartwater sixty-one days previously. But in series 614 it was detected considerably earlier in one larva twenty-seven days after feeding. It was not, however, again seen in this series (614) until forty days after feeding, when the larvae had moulted.

Repeated attempts to bring the Rickettsia to light in both series in this interval were unsuccessful. Nor could the infectivity of the larvae at this time be tested experimentally, because they were engorged with blood, and consequently were not in a condition to feed. It is possible that during this period Rickettsia were present singly or in relatively small aggregates and were rapidly bleached in the differentiation of the strain. The corresponding failure (2) to observe them in the circulating blood of mammals, the infectivity of which was easily proved by sub-inoculation, may be susceptible of a similar explanation.

After the sixty-first day in series 607 and the fortieth day in series 614, the Rickettsia were regularly found in about 75 per cent. of the nymphae examined. It is probable that in series 607 they occurred in small masses before moulting, as in series 614, but escaped recognition.

Contrary to expectations, the Rickettsia seemed to be strictly limited to the epithelial cells of the intestine and to the lumen of the gut. They were never detected with certainty in the cells of the salivary glands. Perhaps in this location also they may have escaped observation, because the accurate identification of a single Rickettsia embedded in cells which are so highly granular, even under the most favourable conditions, would naturally be a problem of considerable difficulty, particularly if the Rickettsia happened to be of spherical shape. But, although infection may take place through the pouring of salivary secretion, containing Rickettsia, into the wound at the time of feeding, this is not necessarily the case. An example of another method of transmission is afforded by the tick *Ornithodorus moubata*. It will be recalled that Leishman has brought forward considerable evidence to show that at ordinary temperatures the salivary glands of *Ornithodorus moubata* do not harbour *Spirochaeta duttoni* and that infection with relapsing fever probably takes place, either by regurgitation or by the excretion of infective matter from the alimentary canal. In either case the Spirochaetes would penetrate through the wound caused by the tick's bite (14).

The experiments made in the tick transmission of the disease have been summarized in Table 4.

TABLE 4.

Summary of experiments showing the infectivity of ticks containing Rickettsia ruminantium.

Ticks of Series.	Fed on.	Produced.
614, renumbered 636	Sheep 8049	After an incubation period of 16 days, an abrupt rise of temperature to 106·8, which was maintained at about 105·8 for for 4 days, then fell to normal and the animal recovered.
607, renumbered 637	Goat 9651	After being held under observation for 19 days, produced no reaction. (This animal was probably immune.)
614, renumbered 638	Goat 9665	After an incubation period of 14 days, a sudden rise of temperature to 107, when the animal was killed.
614, renumbered 640	Goats 8235 8417	In goat 8235, after an incubation period of 13 days, a rise of temperature to 106, which oscillated for 4 days between 106 and 104, when the animal died. In goat 8417, after an incubation period of 18 days, a sudden rise in temperature to 106, which was maintained for 3 days, then it fell to normal and rose again to about 106 on the 25th day. This was in turn maintained for 4 days, when it subsided to normal, and the animal was discharged.
614; the engorged nymphae were not renumbered	Goat 9662	After an incubation period of 12 days, an abrupt rise in temperature to 107·0, which was maintained with slight oscillations for 7 days, then commenced to fall, and the animal died.

In brief, the nymphae which possessed Rickettsia in their alimentary tracts produced typical attacks of heartwater when fed on susceptible sheep and goats, in the tissues of which identical Rickettsia made their appearance. Thus, in ticks, as in mammals, the Rickettsia proved to be inseparable from the virus. Like the virus, they were acquired when larvae were fed on animals suffering from heartwater and containing Rickettsia. Like the virus, also, they passed through the first moulting period into nymphs, in which their presence coincided with the presence of the virus, or, in other words, with the ability to transmit heartwater. And furthermore, it is known that the virus will pass the second moulting period into adults, in which again Rickettsia were found and have been represented in fig. 13.

That *Rickettsia ruminantium* is a very specific micro-organism and is confined to the heartwater tick, *Amblyomma hebraeum*, was observed by an examination of preparations made primarily for other purposes (5, 6) of several other ticks, the names of which follow:—

Amblyomma americana, *Amblyomma maculatum*, *Amblyomma tuberculatum*, *Argas persicus*, *Boophilus decoloratus*, *Dermacentor albipictus*, *Dermacentor variabilis*, *Dermacentor venustus*, *Haemophysalis leachi*, *Haemophysalis leporis palustris*, *Hyalomma aegyptium*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Margaropus winthemi*, *Ornithodorus moubata*, *Ornithodorus megnini*, *Ornithodorus turicata*, *Rhipicephalus appendiculatus*, *Rhipicephalus capensis*, *Rhipicephalus evertsi*, *Rhipicephalus pulchellus*, *Rhipicephalus sanguineus*, and *Rhipicephalus simus*.

Discussion.

In concluding the first section of this paper on heartwater (2), *Rickettsia ruminantium*, as it appeared in the tissues of animals suffering from the disease, was compared with the Rickettsia which are associated with Rocky Mountain spotted fever and with typhus fever as observed in the tissues of man and mammals.

In order to supplement this comparison and to furnish more complete data concerning points of similarity and of dissimilarity between this interesting condition in ruminants and human disease, the following table was prepared, giving the properties of Rickettsia as seen in the arthropod vectors. For convenience, the comparison has been made along the same lines as in Section I, of which it is merely a continuation. The chief difference is that in this case trench fever has also been considered, since the Rickettsia presumably concerned with it are well known in lice. The comparison of *Rickettsia ruminantium* with the *Dermocentroxenus Rickettsia* of spotted fever has been greatly facilitated by the kindness of Dr. R. P. Parker, of Hamilton, Montana, in sending to Dr. Noguchi an abundant supply of ticks infected with the virus of spotted fever, some of which Dr. Noguchi very courteously made available for histological study.

Table 5.

Comparison of the Rickettsia of Rocky Mountain spotted fever, typhus fever, trench fever, and heartwater as seen in the arthropod vectors.

	Spotted Fever: *Dermacentroxenus Rickettsia.*	Typhus Fever: *R. prowazeki.*	Trench Fever: *R. quintana.*	Heartwater: *R. ruminantium.*
Morphology.....	More pleomorphic than in infected mammals— (*a*) extranuclear bacillus - like form without chromatoid granules, 0·5 to 1 μ in length; (*b*) very minute rods with chromatoid granules within nuclei; (*c*) larger lanceolate paired forms (17); "less bacterium-like than any of the Rickettsia" (16); "does not occur in thread-like or filamentous forms as does *Rickettsia prowazeki*" (16)	More pleomorphic than in infected mammals, 0·3 by 0·4 μ for single elements and 3·0 by 0·9 μ for double (13)	Pleomorphic, not definitely known in man (15), 0·3 by 0·3 or 0·3 by 0·5 μ round, oval, diplococci or bacillary with stained holes (18); "more uniform in morphology, plumper and more definitely oval than *Rickettsia porwazeki*" (16); "shorter and thicker than the bacilli of typhus" (18)	More pleomorphic than in infected mammals, cocci 0·2 to 0·3 μ in diameter; bacilli 0·2 to 0·3 by 0·4 to 0·5 μ; and diplococci 0·2 by 0·8 μ no filamentous forms observed.
Phases of multiplication	Intracellular and intranuclear multiplication; "in multiplicative form always shows red and blue staining materials" (16)	"Multiplies exclusively within cells in the louse" also shows (16) red and blue staining materials	Multiplies within the lumen of the intestine of the louse and on the surface of the epithelial cells	In multiplicative form also shows red and blue staining materials; multiplication takes place within the intestinal epithelial cells and perhaps in the intestinal lumen.

	Spotted Fever: *Dermacentroxenus Rickettsia.*	Typhus Fever: *R. prowazeki.*	Trench Fever: *R. quintana.*	Heartwater: *R. ruminantium.*
Microchemical reactions	Gram negative extranuclear bacillary forms stain pale blue with Giemsa; minute rods stain blue, with deeply coloured chromatoid granules; and larger lanceolate paired forms, purple (17), outlines less sharp than most bacteria not acid fast	Gram negative stains feebly by basic aniline dyes pale blue by Giemsa (16), outlines less sharp than most bacteria not acid fast	Gram negative "easier to stain than *Rickettsia prowazeki*" (16), purple or red by Giemsa (16), outlines less sharp than most bacteria not acid fast	Gram negative easily stained dark blue by Giemsa; outlines less sharp than most bacteria not acid fast.
Detection.....	Very simple in well fixed tissues and smears, and much easier than in infected mammals	The same	The same	The same.
Motility.....	Absent	Absent	Absent	Absent.
Position.....	Extracellular, intracellular, intranuclear	Extracellular, intracellular, never intranuclear	Chiefly extracellular (19); never intranuclear	Sometimes extracellular, but chiefly intracellular, never intranuclear.
Arrangement.....	Haphazard; singly, end to end, or in clumps depending upon physical conditions in the tissues	Haphazard: in very large masses	Inlayers upon surface of intestinal epithelium	Densely packed together in very characteristic clumps which are usually spherical and several of which may occur in the cytoplasm of a single cell.
Distribution.....	In all the tissues of the tick in certain phases of the life-cycle, at other times more restricted	In the epithelial cells lining the alimentary tract and in the lumen	Within lumen of alimentary tract	In epithelial cells lining alimentary tract and in lumen.
Effect on host...	Not harmful to ticks	"Eventually causes the death of the louse through suspension of digestion" (16)	Not harmful to lice	Not harmful to ticks.
Infestation.....	Hereditary.....	Not hereditary (16)	Not hereditary (20); hereditarily transmitted (16)	Not hereditary.

This table shows that *Rickettsia ruminantium* as it exists in its arthropod host, resembles the type species, *Rickettsia prowazeki*, rather more closely than does the Rickettsia of Rocky Mountain spotted fever, more particularly in its position within the living epithelial cells of the alimentary tract and in the absence of intranuclear forms. Unlike the other three Rickettsia, filamentous forms were not observed, but, like them, *Rickettsia ruminantium* was

Gram-negative, showed red and blue staining materials on division, possessed rather less sharply outlined contours than most bacteria, and was of very minute size.

Concerning the causative relationship of Rickettsia to these three diseases and to heartwater, there remains, however, an element of uncertainty, because in none of them have the Rickettsia been isolated in pure cultures and the disease produced by inoculation therewith. In the case of Tsutsugamuchi disease, conditions seem to be reversed, inasmuch as Sellards (10) has apparently been successful in cultivating a Rickettsia-like micro-organism which has not thus far been clearly observed in the tissues of patients suffering from the disease or in the arachnids which transmit it (20). His observations furnish a point of departure for the histological examination of the tissues in Tsutsugaamuchi disease and may point the way to the successful cultivation of other pathogenic Rickettsia. In the earlier studies on Rickettsia their resistance to attempts at cultivation upon artificial media was frequently cited as a point of importance in identification. Happily this resistance appears to be giving way first in *Rickettsia melophagi* (21) and *Rickettsia rocha-limae* (22), which most closely approximate to ordinary bacteria in their properties and it is to be hoped, later, in the cases of other Rickettsia which are more aberrant. Indeed, one of the least bacterial in nature, *Dermacentroxenus Rickettsia,* has been successfully propagated in tissue cultures by Wolbach and Schlesinger (23), and *Rickettsia ctenocephalii* has been cultivated in the coelom of the body-louse by Sikora (24).

While the majority of the forty-two species of Rickettsia recently enumerated (11) continue to be uncultivatable and their general properties to be so little known, it is natural that opinions will be constantly changing with the income of new data regarding the proper definition of this important group of micro-organisms. The most radical suggestion recently advanced is that of Hertig and Wolbach (11), who present for consideration the wisdom of restricting the term "Rickettsia" to proved pathogenic micro-organisms having the following characteristics: small size, pleomophism, slight affinity for aniline dyes, and intracellular habitat.

The adoption of this definition would exclude the important and interesting Rickettsia associated in lice with the presence of the virus of trench fever, because they are characteristically extracellular and have only rarely been reported in an intracellular position. Thus Wolbach, Todd, and Palfrey (16) were unable "to find evidence of intracellular distribution or multiplication of the Rickettsia acquired by the stock lice fed upon Mr. Bacot during his illness with trench fever, although twenty-six were examined by serial section." But the definition would include *Rickettsia ruminantium,* although it stains rather more intensely with aniline dyes than does the type species, *Rickettsia prowazeki.* The elimination of *Rickettsia lectularia* of the bed-bug from this group of micro-organisms would also follow (since it is not known to be pathogenic), notwithstanding the fact that it resembles *Rickettsia prowazeki* as closely, or perhaps more so, than does *Dermacentroxenus Rickettsia.* It is, moreover, of small size, very pleomorphic and exhibits but slight affinity for aniline dyes. It is Gram-negative and is typically an intracellular parasite. In short, *Rickettsia lectularia* is one of the most characteristic of the Rickettsia, under which heading it is firmly established in the literature.

In a recent paper Arkwright (25) expressed the opinion that Rickettsia are only known to be concerned with three diseases: Rocky Mountain spotted fever, typhus fever, and trench fever. To these three we may add heartwater, and possibly, as a result of Sellard's work, Tsutsugamuchi disease also. If the observations reported in this paper are confirmed, the recognition of typical Rickettsia in heartwater will enlarge our conception of the scope of the pathogenicity of these micro-organisms, which has hitherto been restricted to man and experimental aminals, by the inclusion of a disease of domestic animals which is never found in man. The bringing of new diseases under the general heading of " Rickettsiosis " suggests the likelihood that the limitations of this group have not yet been established and that other febrile diseases of unknown etiology, which are insect-transmitted and of which there are several, particularly in South Africa, may eventually be found to fall in the same category. It is not too much to hope that with the enlargement of the group and the acquisition of detailed information concerning its constituent members, our fundamental knowledge of the nature of Rickettsia-like micro-organisms will be advanced.

Results.

The evidence observed in favour of a causative relationship between *Rickettsia ruminantium* and the production of heartwater falls under the following headings:—

(1) The discovery of *Rickettsia ruminantium* in the tissues of goats, sheep, and cattle suffering from heartwater; the close association between their presence in these three species and the febrile reaction; their disappearance soon after the fever has commenced to decline corresponding to the loss in infectivity of the blood; and their absence in control animals (Part 1 of this Report).

(2) The appearance of the same Rickettsia, easily identifiable by their very characteristic morphology and staining reactions in two series of ticks (607 and 614) which had fed upon cases of heartwater and which, before feeding, did not contain them; and their non-appearance in three series of control ticks (604, 605, and 606) from the same parent stock which had fed upon normal animals.

(3) The fact that the ticks containing Rickettsia in their alimentary tracts when fed upon susceptible animals, produced in them typical attacks of heartwater, which the control ticks, devoid of Rickettsia, failed to do.

(4) The completion of the cycle by the recognition of the same Rickettsia in the tissues of these animals (sheep 8049 and goat 9651) which contracted heartwater as a result of having been bitten by the infective ticks containing Rickettsia.

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DESCRIPTION OF FIGURES.

PLATE I.

All the figures in Plate I were drawn with Zeiss apochromatic objective 1.5 mm., compensating ocular 8, and camera-lucida, and have been reproduced without reduction, so that they represent a magnification of 1,940 diameters.

Fig. 1 illustrates Rickettsia composed of two materials, one coloured faint red or purple and the other light blue. From a smear of a nymph of Series 614 made 63 days after the nymph had commenced to feed as

a larva on Bull 424 suffering from heartwater. The smear was dried in air, fixed in absolute alcohol, and coloured by Giemsa's stain.

Fig. 2.—Large clump of Rickettsia from a section of a nymph of series 607, made 63 days after feeding on Calf 928 suffering from heartwater; fixed in Regaud's fluid and coloured by Giemsa's stain.

Fig. 3.—Clumps of Rickettsia in a smear of nymph of Series 614 made 58 days after feeding on Bull 424; fixed in absolute alcohol and coloured by Giemsa's stain.

Fig. 4.—(*a*), (*b*), (*c*) Three clumps of Rickettsia in intestinal epithelial cells showing certain larger more irregular bodies stained dark red. From a section of a nymph of Infective Series 607 fixed, 77 days after feeding, in Regaud's fluid and coloured by Giemsa's stain. (*d*) A single clump of Rickettsia embedded in a blue-stained matrix. From a section of a nymph of Infective Series 607 fixed, 79 days after feeding, in 10 per cent. formalin and coloured by Giemsa's stain. (*e*) Another clump of more lightly coloured Rickettsia within a blue-stained matrix. From a section of a nymph of Infective Series 607 fixed, 88 days after feeding, in Regaud's fluid and coloured by Giemsa's stain. (*f*) and (*g*) Two clumps of Rickettsia swollen by the action of formalin. From the same preparation as (*d*).

Fig. 5.—Two clumps of Rickettsia within an intestinal epithelial cell. From a nymph of Infective Series 607 fixed, in Regaud's fluid 69 days after feeding, and coloured by Giemsa's stain.

Fig. 6.—Large intestinal epithelial cell containing several clumps of micro-organisms. From a nymph of Infective Series 607 fixed, in Regaud's fluid 64 days after feeding, and coloured by Giemsa's stain.

Fig. 7.—A portion of the wall of a Malpighian tubule above, and below adjacent epithelial cells of the intestine. The cells of the Malpighian tubule are infested by large bacillary micro-organisms, and the intestinal cells contain clumps of Rickettsia. Neither of the two types of cells show signs of injury resultant upon the presence of the micro-organisms. From a nymph of Infective Series 607 fixed, in Regaud's fluid 70 days after feeding, and coloured by Giemsa's stain.

PLATE II.

The photomicrographs were taken with Zeiss apochromatic objective 3 mm., 1.40 aperture, and compensating ocular 8, giving a magnification of 1,400 diameters.

Fig. 8.—Two clumps of Rickettsia, one spherical, very densely packed and to the left, the other slightly to the right of the centre and showing well the individual Rickettsia. From a nymph of Infective Series 607 taken 84 days after feeding as a larva; fixed in 10 per cent. formalin and stained by Goodpasture's fuchsin method.

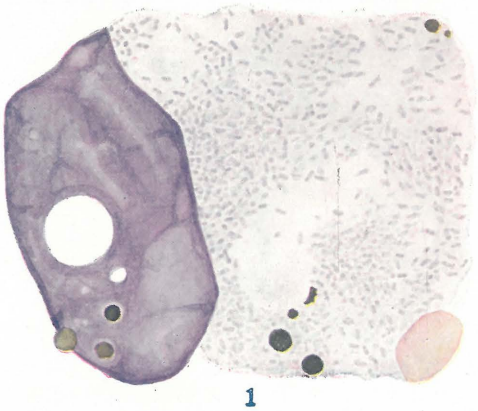
Fig. 9.—Intestinal epithelial cells containing one very large and deeply stained oval clump of Rickettsia to the left, a somewhat smaller one to the right and still smaller, scattered, irregular masses of Rickettsia. From a nymph of Infective Series 614 taken 67 days after feeding; fixed in Regaud's fluid and coloured by Giemsa's stain.

Fig. 10.—Clumps of Rickettsia in a large intestinal epithelial cell. Close examination reveals the presence of rather larger atypical bodies (see page 188) embedded among the Rickettsia. From a nymph of Infective Series 607 taken 78 days after feeding as a larva and fixed in Regaud's fluid and coloured by Giemsa's stain.

Fig. 11.—Two clumps of Rickettsia within the lumen of the rectal sac. From a nymph of Infective Series 607 taken 84 days after feeding as a larva; fixed in Carnoy's 6 : 3 : 1 fluid and coloured by Giemsa's stain.

Fig. 12.—Large intestinal epithelial cell containing several clumps of Rickettsia embedded in a highly granular cytoplasm. From a nymph of Infective Series 607 which had fed on Goat 9651 and had been renumbered 637 (see Table 4). The viscera of the nymph were fixed in Regaud's fluid and coloured by Giemsa's stain.

Fig. 13.—Clumps of Rickettsia with an intestinal epithelial cell in an adult descended from a larva which 96 days previously fed on an animal suffering from heartwater and which, as a nymph, fed upon Goat 9665, transmitting heartwater to it. Fixed in Regaud's fluid and coloured by Giemsa's stain.



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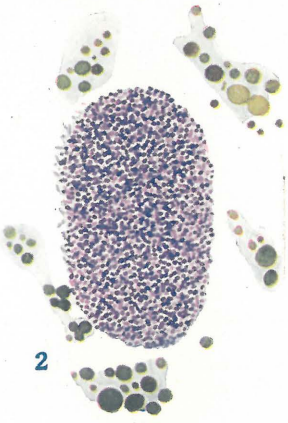

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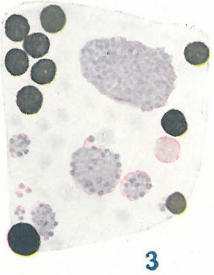

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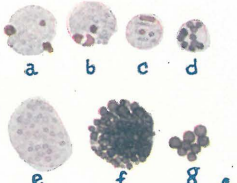

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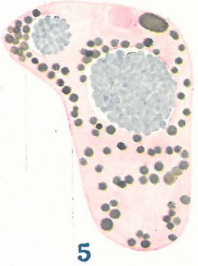

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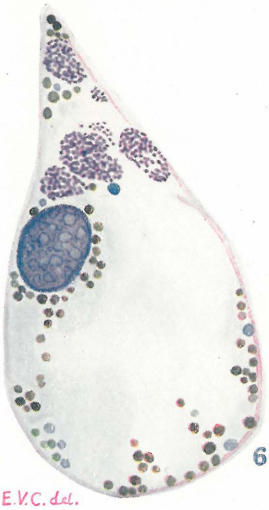

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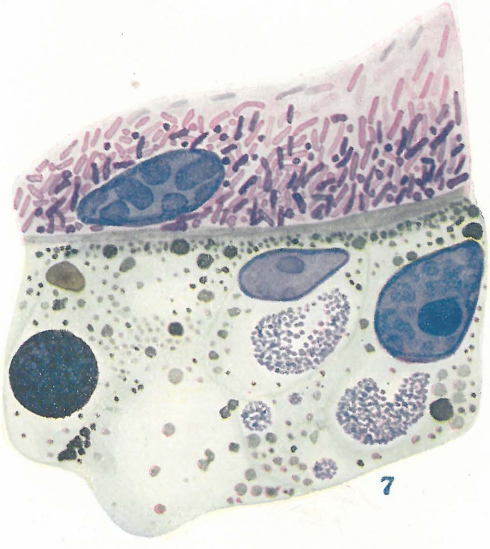

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E.V.C. del.

PLATE I.

Heartwater II.] [*E. V. Cowdry.*

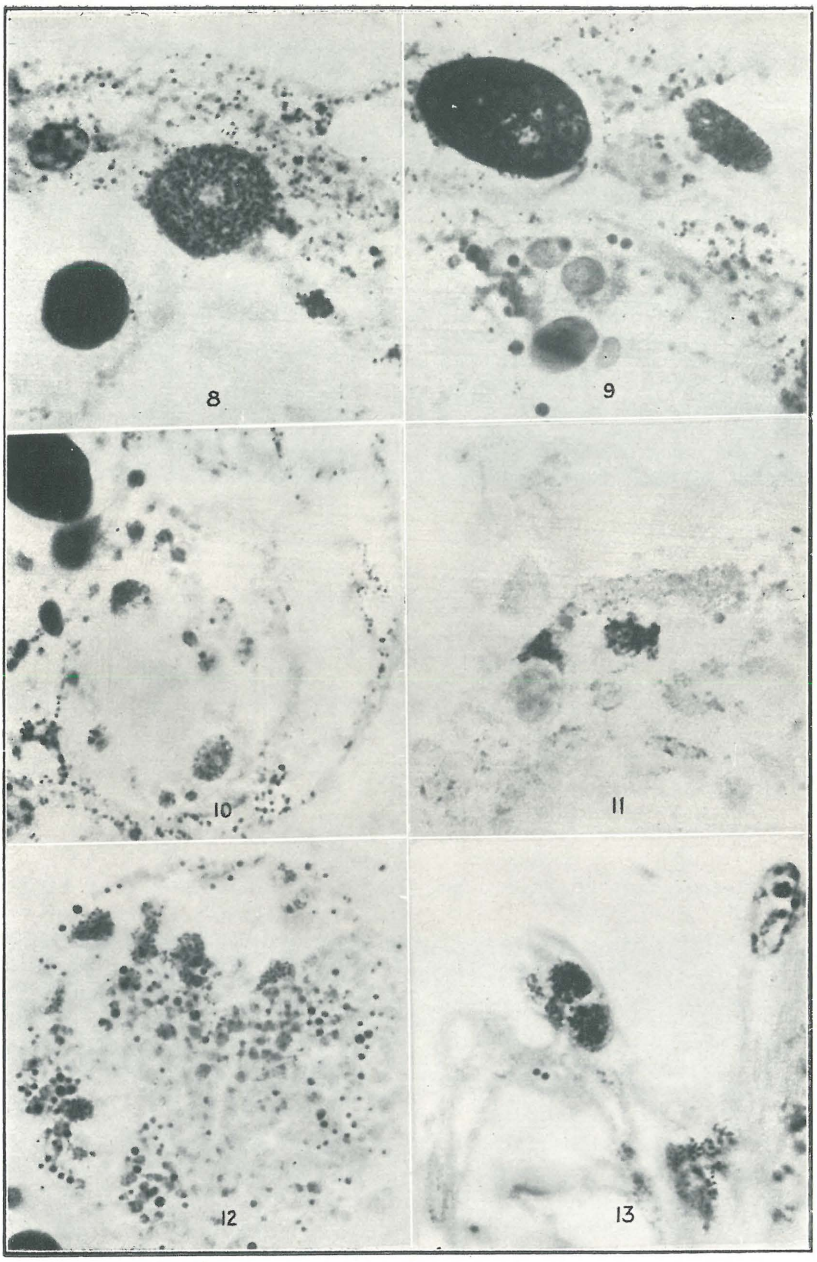

Plate II.] [*E. V. Cowdry.*

HEARTWATER II.