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Mots-clés : Microorganismes de type rickettsien. *Borrelia burgdorferi. Ixodes ricinus.* Développement des ovaires.

RICKETTSIA-LIKE MICROORGANISMS IN THE OVARIAN PRIMORDIA OF MOLTING *IXODES RICINUS* (ACARI : IXODIDAE) LARVAE AND NYMPHS

Z. ZHU*, A. AESCHLIMANN**, L. GERN**

SUMMARY

A large number of coccoid, or oval to spindle-shaped rickettsia-like microorganisms are detected intracellularly within the primordial ovaries of all examined molting larvae and nymphs, descendants of a female *Ixodes ricinus* collected in a forest near Neuchâtel, Switzerland, and within all molting nymphal *I. ricinus* captured as starved ticks in the same area. The morphology of the microorganisms is identical in all ticks. They measure 0.3-0.4 μm in diameter and 0.6-1.3 μm, occasionally up to about 2.5 μm in length. The microorganisms possess a highly rippled membranous outer cell wall and an inner plasma membrane. Both cellular boundaries show a thickness of approximately 6-10 nm. The microorganisms are always enclosed, singly or in groups, within membrane limited vacuoles of host cells, within vacuolated mitochondria, or within vacuoles with their wall consisting partially of a deformed mitochondrion and partially of a plasma membrane of host cells. With the advance of the development of the nymphal ovaries, the groups of microorganisms associated with mitochondria become larger and increase in number, and they are dominant within the posterior part of the organs in the nymphs examined at day 21 after repletion. The relationship between the microorganisms and the mitochondria is discussed. The morphology of the gonads of the examined immature ticks is also described.

RÉSUMÉ : **Micro-organismes de type rickettsien dans les ébauches des ovaires de larves et de nymphes d'*Ixodes ricinus* en train de muer.**

Un grand nombre de micro-organismes de type rickettsien ont été observés dans les cellules des ébauches ovariennes de larves et de nymphes d'*Ixodes ricinus,* lors de la mue. La morphologie de ces organismes est identique chez toutes les tiques examinées. Ils mesurent 0,3-0,4 μm de diamètre et 0,6-1,3 μm, parfois jusqu'à 2,5 μm, de long. Ils possèdent une paroi externe membraneuse fortement ondulée et une membrane plasmique interne. L'épaisseur de chacune de ces 2 structures est de 6-10 nm. Les micro-organismes sont toujours inclus, soit seuls soit en groupe, dans des vacuoles contenues dans le cytoplasme de la cellule hôte, dans des mitochondries vacuolées, ou dans des vacuoles dont les parois sont constituées en partie de restes de mitochondrie. Plus les ovaires des nymphes sont développés, plus le nombre de micro-organismes associés aux mitochondries augmente. La relation entre les micro-organismes et les mitochondries est discutée. la morphologie des gonades chez les tiques immatures est décrite.

INTRODUCTION

Rickettsia-like microorganisms (RMs), grouped in the genus *Wolbachia* (Philips, 1963), are generally considered to be symbiotes of arthropods and non-pathogenic for man and animals. They have been reported in ticks since a long time (Roshdy, 1961*a*), and were found intracellularly in the different tissues of argasid (Cowdry, 1925; Mudrow, 1932; Suitor and Weiss, 1961; Roshdy, 1961*b*, 1964*a*, 1964*b*, 1964*c*; Balashov, 1968; Hecker *et al.*, 1968; Aeschlimann and Hecker, 1970; Reinhardt *et al.,* 1972; Burgdorfer *et al.,* 1973; Diehl and Aeschlimann, 1982; El Shoura, 1985; 1986*a*; 1986*b*; 1987; 1988; 1990; El Shoura and Roshdy, 1985; El Shoura *et al.,* 1989) and ixodid ticks (Cowdry, 1925; Mudrow, 1932; Roshdy, 1961*a*; Balashov, 1968; Lewis, 1977, 1979; Šuťáková and Řeháček, 1991). Amongst these numerous investigations, RMs were reported within mitochondria only in the ovaries of *Ixodes ricinus* females from a laboratory strain in England (Lewis, 1977, 1979). Our recent ultrastructural investigations into the development of *Borrelia burgdorferi (Spirochaetales: Spirochaetacea)* in *I. ricinus* showed that the primordial ovaries of molting larvae and nymphs were infected with a great number of RMs. They were also found in association with mitochondria. In the molting nymphs systemically infected

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with *B. burgdorferi,* the primordial ovaries were concurrently infected with both microorganisms (Zhu *et al.,* 1991*b*; 1991*c*). This paper describes the distribution and ultrastructure of the RMs, as well as the morphology of the gonads of the examined molting immature ticks.

MATERIALS AND METHODS

Adult and nymphal *I. ricinus* were collected by flagging vegetation in a forest near Neuchâtel, Switzerland. Larvae and nymphs at week 20 after hatching or molting, descendants of a wild-collected female engorged on an uninfected New Zealand white rabbit (Graf, 1978), were allowed to feed on an uninfected New Zealand white rabbit (Graf, 1978). Wild-captured nymphs were allowed to engorge on an additional New Zealand white rabbit. The repleted ticks were held at 20-22° C and saturated humidity. The laboratory-obtained larvae and nymphs were dissected in phosphate buffered saline at day 13 (n = 8) and 21 (n = 5) after repletion, respectively. The wild-collected nymphs were dissected at day 15 (n = 8) and 21 (n = 4) after repletion. The larvae with their partial lateral body cut off and the nymphs halved longitudinally were fixed in freshly prepared Karnovsky's fixative (Karnovsky, 1965; Zhu *et al.,* 1991*a*), and were processed for transmission electron microscopy (Agbede *et al.,* 1986; Zhu *et al.,* 1991*a*, 1991*b*, 1991*c*).

RESULTS

MORPHOLOGY OF THE GONADS OF MOLTING LARVAE EXAMINED AT DAY 13 AFTER REPLETION

Only the posterior arched part of the gonads was observed. It is situated transversally over the rectal tube and below the posterior end of the stomach. This location is similar to that of the larval gonads of *Argas persicus* (Balashov and Goroshchenko, 1960; Roshdy, 1964*a*) and of *Rhipicephalus appendiculatus* (Till, 1961), but a little more onward. The longitudinal sections of the posterior arched part show that the gonads do not have a lumen and are only one to two cell wide, though the cellular limits are quite distinguishable *(Figs. 1-3).* They are covered by a delicate layer of connective tissue. In the gonads two types of nuclei can be recognized : large oval or spherical nuclei and smaller oval or angular ones rich in chromatin. The former are assumably the nuclei of genital cells and the latter are those of the supporting cells (Mudrow, 1932; Balashov and Goroshchenko, 1960; Roshdy, 1964*a*; Till, 1961; Balashov, 1968).

In the ovarian primordium of the female molting larvae, the small nuclei of the supporting cells are scattered very sparsely along the peripheral region. Thus, in the longitudinal sections, the gonad consists mostly of the large genital cells that are located in the central part of the gonad *(Figs. 1-2).* The cytoplasm of both types of cells possess many lysosome-like multi-vesicular vacuoles and vacuolated mitochondria *(Figs. 1, 2, 4).* The posterior end of the ovarian primordium is up to about 9 μm in diameter *(Figs. 1-2).*

In the testicular primordium, although the small nuclei of the supporting cells are also distributed in the peripheral region of the gonad and the large nuclei of the genital cells are mostly located in the central part of the organ, the two kinds of nucleus appear to be intermixed and the small nuclei of the supporting cells are much more nume-

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FIG. 1. — Longitudinal ultra-thin section through the arched posterior part of the primordial ovary of a larva at day 13 after repletion, demonstrating numerous RMs (= R), most of which are single, in the cytoplasm of the ovarian cells. Many electron dense lysosome-like multi-vesicular vacuoles (L) with different sizes and two kinds of nuclei of ovarian cells can be seen. The large centrally situated nuclei are the nuclei of genital cells (Ng) and the small periphal one rich in chromatin is that of the supporting cell (Ns). C connective tissue surrounding the ovary; H. hemocoel; M mitochondria; Ws wall of the posterior end of the stomach. Electron micrograph. Bar = 3 μm.

FIG. 2. — Longitudinal ultra-thin section through the posterior arched part of the primordial ovary of a larva at day 13 after repletion, demonstrating numerous RMs (= R), electron dense autolysosome-like multi-vesicular vacuoles (L) of varying sizes and several deformed or vacuolated mitochondria (M). Note two large nuclei of genital cells (Ng) and the definite cell limits (arrowheads). One of the vacuolated mitochondria housing several rickettsia-like microorganisms in the centre of this figure is shown in detail in Fig. 4. Four degenerating RMs (= Dr) are shown at the top left. C connective tissue of the ovary. Electron micrograph. Bar = 2 μm.

FIG. 3. — Longitudinal ultra-thin section traversing the posterior part of the testicular primordium from a larva at day 13 after repletion, showing the location of the testis and two kinds of nucleus and their arrangement within the organ. Note that the testicular primordium is situated below the posterial end of the stomach (Ws) and over the rectal tube (Wr). A peritrophic membrane (Pm) is present in the stomach lumen. The large rounded nuclei of genital cells (Ng) are mainly situated in the central part of the organ, and the small irregularly shaped ones of the supporting cells (Ns) rich in chromatin, are located peripherally. The two kinds of nucleus are intermixed. T testicular primordium; Ws wall of the posterior part of the stomach; Wr wall of the rectal tube. Electron micrograph. Bar = 6 μm.

FIG. 4. — Enlarged view of a MMLV shown in Fig. 2. Note that its wall consists partially of a deformed mitochondrion (double small arrowheads) and partially of a membrane (double small arrows) of host cell. The enclosed RMs (= R) possess a rippled outer cell wall. A reticulated cytoplasmic matrix is present in the microorganisms (big arrow). Note several small rounded vesicles (small arrows) adhering to the outer surface of microorganisms and several fine long tubules (small arrowheads) with an undulate appearance. A short piece of a tubule is in contact with the surface of the deformed mitochondrion (big arrowhead). Electron micrograph. Bar = 0.25 μm.

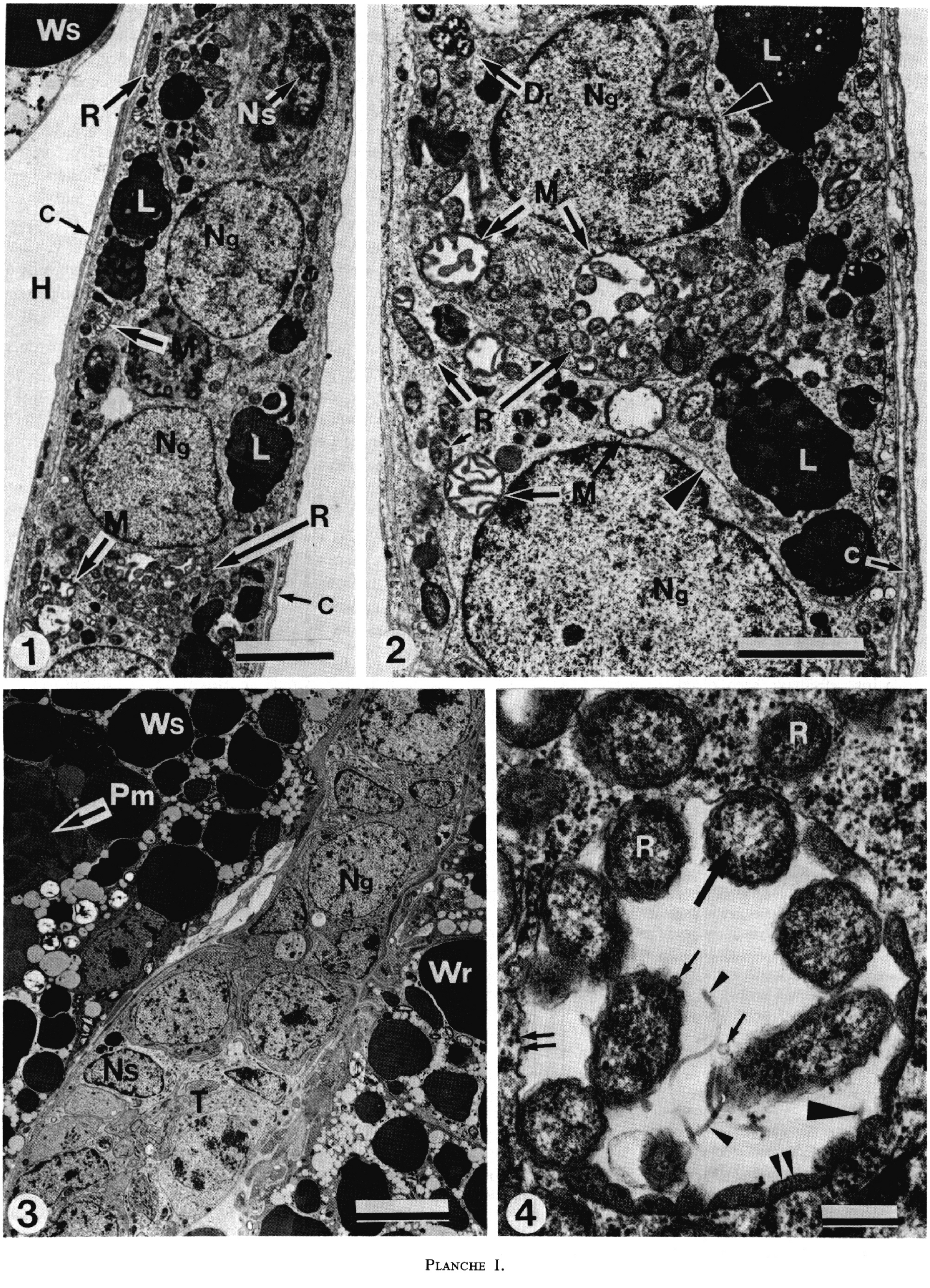

PLANCHE I.

rous than those in the primordial ovaries of the female larvae *(Figs. 1-3)*. The cytoplasm of both types of cells is rich in non-vacuolated mitochondria and the lysosome-like multi-vesicular vacuoles are rarely seen. The posterior end of the testicular primordium measures up to about 15 µm in diameter *(Fig. 3)* and is apparently thicker than that of the ovarian primordium *(Figs. 1-2)*.

RMs are present without exception in the ovarian primordia *(Figs. 1-2)* but are never detected in the testicular primordia of larval ticks.

MORPHOLOGY OF THE GONADS OF MOLTING NYMPHS EXAMINED AT DAY 15 AND 21 AFTER REPLETION

The gonads of the molting nymphs examined at day 15 and 21 after repletion have distinctly differentiated sexually and can be easily recognized in both ultra-thin and semi-thin sections *(Figs. 5-9)*. The cellular boundaries of the gonads have been definite *(Figs. 6-8)*, and two kinds of nucleus similar to those observed in larval gonads are also present.

The ovarian primordium of the female molting nymphs is a horseshoe-like structure and tends to be smaller gradually towards its anterior parts. In the transverse sections, it is rounded. Sections from the posterior part of the primordial ovaries show that the female gonads in the nymphs examined at day 15 after repletion is obviously less developed than those in the nymphs examined at day 21 after repletion. In the former, although most small nuclei of supporting cells have been disposed in the central part, they are still, in some degrees, mixed with the peripheral large nuclei of genital cells and a central ovarian lumen has not occurred *(Fig. 5)*. The posterior end of the ovaries measures up to about 60 µm in diameter *(Fig. 5)*. Whereas in the latter, the central ovarian lumen has appeared and, in some ticks, it has been quite large *(Fig. 8)*. It is surrounded by many small nuclei of epithelial cells, derived from supporting cells, and the large oogonia or oocytes lie peripherally *(Fig. 8)* (Balashov, 1968). The posterior end of the ovaries measures up to about 75 µm and is evidently thicker than that in the former. Like in the female larvae mentioned above, many lysosome-like multi-vesicular vacuoles are seen in the cytoplasm of the ovarian cells of these two groups of molting nymphs, and many mitochondria located in the ovaries are vacuolated *(Figs. 6-8)*.

The testicular primordium of the male molting nymphs is apparently thicker than the ovarian primordium of the female ones, and it measures up to about 190 µm in diameter in nymphs examined at day 15 after repletion *(Fig. 9)*. It consists of two elongated testes fused at their posterior ends. The testes become narrow anteriorly and consist of spermatocysts covered with epithelial cells, derivatives of supporting cells *(Fig. 9)* (Till, 1961; Balashov, 1968; Oliver and Briton, 1972). The cytoplasm of the spermatocytes within the spermatocysts is rich in non-vacuolated mitochondria and the lysosome-like multi-vesicular vacuoles are seldomly found. The testicular primordia of the nymphs examined at day 15 and 21 after repletion resemble each other.

RMs are observed within the primodial ovaries of all female molting nymphs *(Figs. 6-8)*. They have never been found within the testicular primordia of male ones.

PLANCHE II.

Fig. 5. — Longitudinal semi-thin section through the posterior part of the primordial ovary from a nymph at day 15 after repletion, exhibiting that the small irregularly shaped and chromatin-rich nuclei of supporting cells (Ns) are located centrally, and that the large rounded nuclei of genital cells (Ng) are situated peripherally. Note that the ovary lacks a central lumen and tow kinds of nucleus are mixed in some degrees at the central part of the ovary. H hemocoel; W midgut wall. Optical micrograph, toluidine blue. Bar = 20 µm.

FIG. 6. — Transversal ultra-thin section crossing the posterior part of the primordial ovary from a nymph at day 15 after repletion, showing numerous RMs (= R) within the ovarian tissue. Note that a large groupe of RMs and numerous irregularly-shaped electron-lucent vesicles (small arrow) are within a membrane-limited vacuole (V). Also note the singly scattered RMs, and several vacuolated mitochondria (M). One of the mitochondrion at the lower right harbours a small group of the microorganisms. A *B. burgdorferi* (B) in the extracellular space of the connective tissue (C) surrounding the ovary. H hemocoel; L lysosome-like multi-vesicular vacuole. Electron micrograph. Bar = 2 µm.

FIG. 7. — Transversal ultra-thin section of the ovary from a nymph at day 15 after repletion, showing intracellular RMs (= R) in the ovarian tissue and extracellular *B. burgdorferi* (B). A degenerating fissioning RM (= Dr) is shown at the lower left. Note the large electron-dense areas suspended in the cytoplasmic matrix and a thick electron-dense layer along the inner side of the inner plasma membrane in this microorganism. The degenerating fissioning form is associated with many lysosome-like vesicles (big arrowhead) and the fission site can clearly be seen (big arrow). L lysosome-like multi-vesicular vacuole. Electron micrograph. Bar = 0.5 µm.

FIG. 8. — Transversal ultra-thin section of the posterior end of the ovarian primordium from a nymph at day 21 after repletion, demonstrating the distribution of RMs (= R) in the organ. Note that most microorganisms are grouped and that most grouped microorganisms are located within vacuolated mitochondria or MMLVs in the cytoplasm of both supporting and genital cells. H hemocoel; L lysosome-like multi-vesicular vacuoles; Lu lumen of the primordial ovary; Nc nucleus of the connective tissue; Ng nucleus of genital cell; Ne nucleus of epithelial cell. Electron micrograph. Bar = 6 µm.

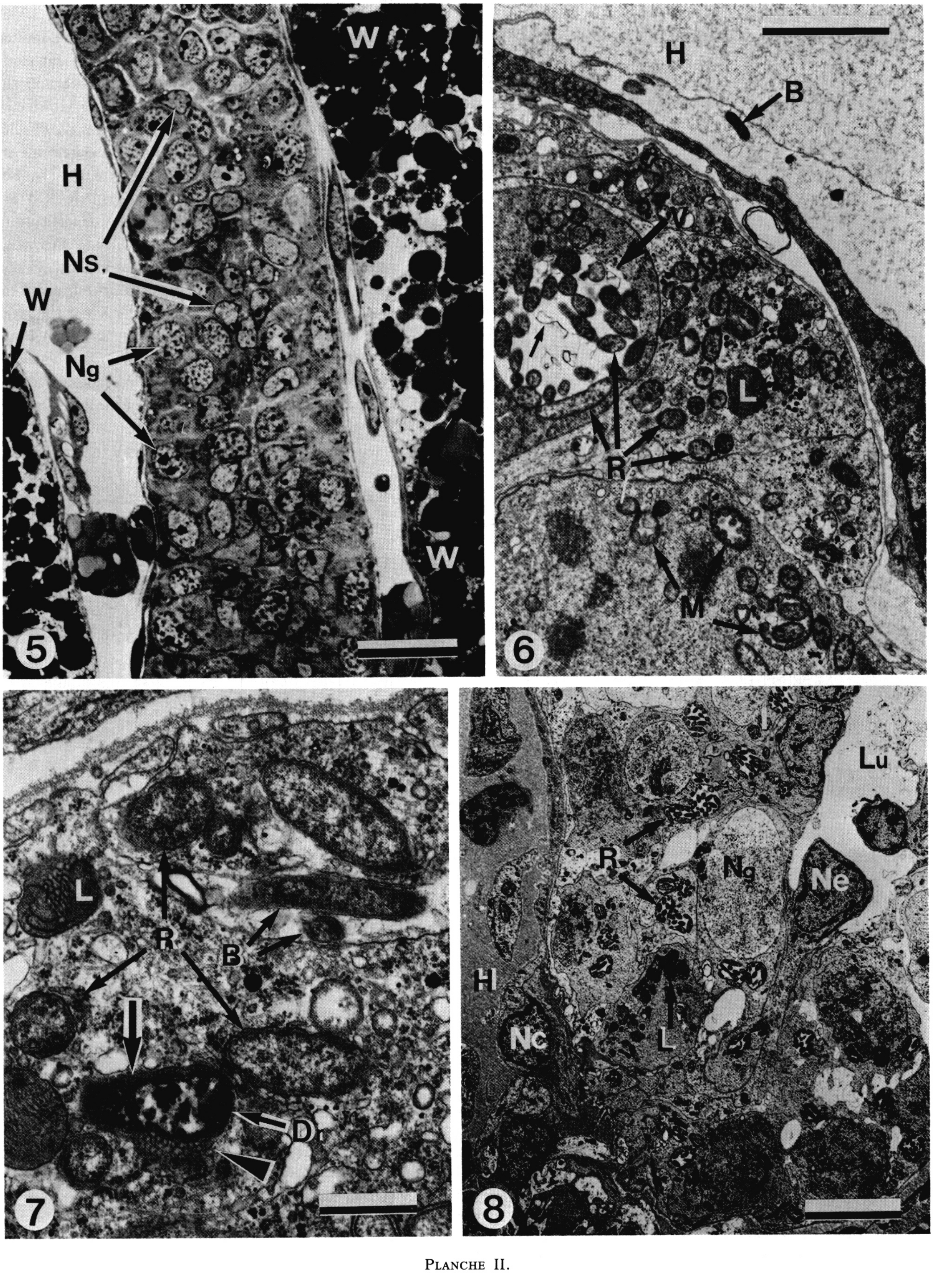

PLANCHE II.

ULTRASTRUCTURE OF RMs IN PRIMORDIAL OVARIES OF MOLTING LARVAE AND NYMPHS

A large number of coccoid, or oval to spindle-shaped RMs are found intracellularly within the primordial ovaries of all examined female molting larvae and nymphs *(Figs. 1-2, 4, 6-8, 10-14)*. The morphology of the RMs is identical in all ticks. They measure 0.3-0.4 μm in diameter and 0.6-1.3 μm *(Figs. 1-2, 4, 6-8, 10-14)*, occasionally up to about 2.5 μm in length *(Fig. 13)*. The cell of the RMs is enclosed with two electron-dense membranous layers: a highly rippled outer membranous cell wall and an inner plasma membrane *(Figs. 4, 10-14)*. They have a same thickness of approximately 6-10 nm *(Figs. 4, 10-14)*. Both membranous layers are three-layered and of typical unit membrane appearance *(Figs. 10-12)*. The inner plasma membrane encloses a cytoplasmic matrix interspersed with electron-lucent spaces or splotches *(Figs. 4, 7, 10-14)*. The ribosomes are the most prominent organelles *(Figs. 4, 7, 10-14)*. A reticulated cytoplasmic matrix is sometimes present *(Figs. 4, 12, 15)*. Transverse binary fissions of the RMs are occasionally observed *(Fig. 7)*. Generally, the dividing forms are seen simply to pull apart into « leaflets » structures (Wright and Barr, 1980) and do not form a septum of cell wall material. However, in a degenerating dividing RM, a cell wall-like septum is observed *(Fig. 7)*. The degenerating forms are mainly found in the degenerating ovarian cells and are enclosed in the lysosome-like multi-vesicular vacuoles *(Figs. 7, 15-16)*. They appear to have condensed their cytoplasm into large electron-dense areas suspended in the cytoplasmic matrix and/or into an electron-dense thick layer along the inner plasma membrane *(Figs. 7, 15)*. In a large lysosome-like multi-vesicular vacuole, the degenerating RMs have only one large electron-dense cytoplasmic area and many filamentary structures suspended in the wide electron-lucent cytoplasmic area *(Fig. 16)*.

The RMs are enclosed, singly or in groups, within membrane-limited vacuoles *(Figs. 6, 10-13)*, within vacuolated mitochondria *(Fig. 14)*, or within vacuoles with their wall consisting partially of an elongated mitochondrion and partially of a plasma membrane of the host cells, which are tentatively named as mitochondrion-membrane-limited vacuoles and abbreviated as MMLVs in the present study *(Figs. 4, 13, 14)*. The RM-infected mitochondria and the mitochondria occupying part of the wall of RM-infected MMLVs appear to be greatly deformed *(Figs. 2, 4, 6, 8, 13-14)* (Lews, 1977: 1979), and sometimes they are not easy to be identified at first glance *(Figs. 2, 4, 6, 8, 13-14)*. The membrane-limited vacuoles enclosing the single RM are usually difficult to be recognized at low magnification, owing to their close association *(Figs. 1-2, 6-8)*. But when high magnification is used, these vacuoles are always distinguishable *(Figs. 10-12)*. Two kinds of electron-transparent vesicles are present in association with the RMs: the large and irregularly shaped vesicles (approximately 50-400 nm in diameter) are seen within the membrane-limited vacuoles of the molting nymphs at day 15 after repletion *(Fig. 6)*; and the small and regularly rounded ones (approximately 20-50 nm in diameter), many of which are in contact with the rippled outer cell wall of the RMs, are located mostly within vacuolated mitochondria *(Fig. 14)* or MMLVs *(Fig. 4)* in all female ticks. Electron-lucent to moderately dense fine tubules are sometimes found together with the small rounded vesicles in the infected mitochondria *(Fig. 14)* and MMLVs *(Fig. 4)*. They appear to be very long (a length of no less than 0.67 μm is measured, *Fig. 4*), undulate, and have an even width of approximately 14-20 nm *(Figs. 4, 14)*. The contact of these fine tubules with the enclosing vacuolated mitochondria *(Fig. 4)* and the adhesion of the regularly rounded small vesicles to these fine tubules *(Fig. 14)* are observed.

DISTRIBUTION OF RMs IN PRIMORDIAL OVARIES OF MOLTING LARVAE AND NYMPHS

Mainly the posterior part of the gonads of the immature ticks was examined for RMs. The RMs are very numerous and are present in every cell of the primordial ovaries of the molting female larvae and nymphs *(Figs. 1-2, 6-8)*.

In the ovarian primordia of the molting larvae examined

PLANCHE III.

FIG. 9. — Transversal semi-thin section of the middle part of a testis (T) from a nymph at day 15 after repletion, showing the testis full of developing spermatocysts (Sp) that are covered with epithelial cells (E). W midgut wall. Optical micrograph, toluidine blue. Bar = 25 μm.

FIG. 10. — Ultra-thin section of a RM within the ovarian tissue of a larva at day 13 after repletion, showing the highly rippled outer cell wall (Cw) and the inner plasma membrane (Mb) of the microorganism, and the enclosing membrane-limited vacuole (V). Note the prominent ribosomes (Rb) in the cytoplasmic matrix. Electron micrograph. *Bar = 0.2 μm*.

FIG. 11. — Ultra-thin section of two RMs enclosed within a membrane-limited vacuole (V) within the ovarian tissue from a nymph at day 21 after repletion. Note the outer rippled cell wall (Cw), the inner plasma membrane (Mb) and the prominent ribosomes (Rb) in the cytoplasmic matrix. Electron micrograph. Bar = 0.2 μm.

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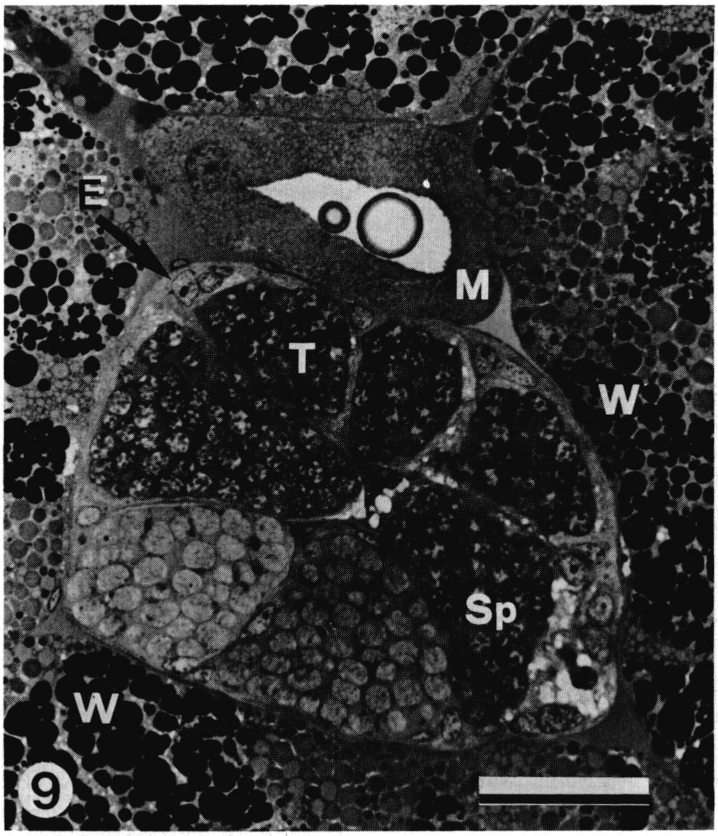

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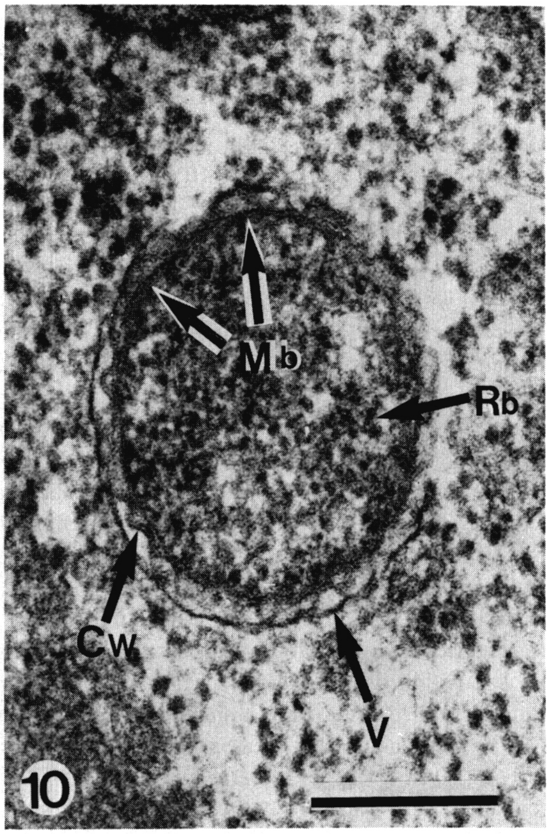

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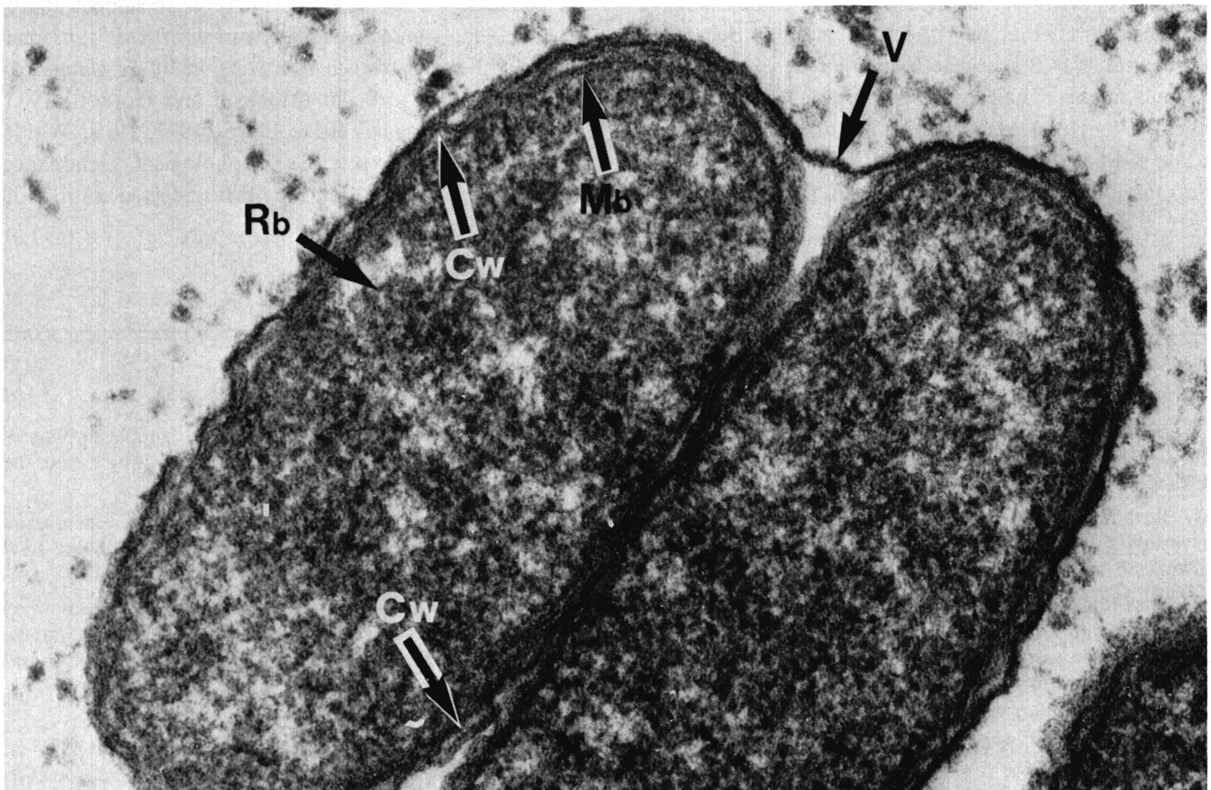

PLANCHE III.

at day 13 after repletion, the majority of the RMs are single and only a small part of them are grouped *(Figs. 1-2)*. Most grouped bacteria are situated within the vacuolated mitochondria or within the MMLVs *(Fig. 2)*. Only one to about 10 RMs can be found in a section of a vacuolated mitochondria, or of a MMLV *(Figs. 2, 4)*.

In the ovarian primordia of the molting nymphs examined at day 15 after repletion, also only a small part of the RMs are grouped, but most grouped forms are contained within large membrane-limited vacuoles *(Fig. 6)*. These vacuoles are located in the peripheral region of the ovarian primordia *(Fig. 6)*. They measure up to 2.5-3.2 μm in diameter and as many as about 40 RMs can be seen in a section of such a vacuole. A few grouped RMs are enclosed within the vacuolated mitochondria *(Fig. 14)* or within the MMLVs *(Fig. 6)*. One to about 10 bacteria are present in a section of the vacuolated mitochondria or MMLVs *(Figs. 6, 14)* .

In the ovarian primordia of the molting nymphs examined at day 21 after repletion, the majority of the RMs are grouped *(Fig. 8)*. Most of these multiple forms are enclosed within the MMLVs, or within vacuolated mitochondria *(Fig. 8)*, and only a small part of the grouped RMs are contained within membrane-limited vacuoles. Up to about 30 RMs can be observed in a MMLV, or in a vacuolated mitochondrion. Although the mitochondria are further deformed, their density within the ovarian tissue of the nymphs examined at day 21 after repletion appears to remain the same as that in the female larvae examined at day 13 and in the female nymphs examined at day 21 after repletion. No difference in the distribution of the RMs in the primordial ovaries was found between the laboratory-obtained and the wild-captured nymphs.

In two nymphs, examined at day 15 and 21 after repletion respectively, the primordial ovaries are concurrently infected with *B. burgdorferi (Figs. 6-7)* (Zhu *et al.*, 1991*b*, submitted).

DISCUSSION

The RMs detected within the mitochondria in the ovaries of the bloodfeeding female *I. ricinus* from a laboratory strain in England were considered to be probably *Ehrlichia phagocytophila* (Ristic and Huxsoll, 1984; = *Cytoecetes phagocytophila,* Foggie, 1962; Lewis, 1977; 1979), the causative agent of tick-borne fever (Lewis, 1977; 1979). Our observations show that the RMs within the ovarian primordia of the immature descendants of a wild-collected *I. ricinus* female and the nymphal *I. ricinus* captured in nature are similar in appearance to those described by Lewis, though they were also found, singly or in groups, within membrane-limited vacuoles or MMLVs, in addition to those seen within mitochondria. Furthermore, we have also found numerous RMs of the same morphology as those detected in the molting larvae and nymphs, within the ovaries in each of 20 wild-collected female *I. ricinus* examined before, during or after bloodfeeding on uninfected New Zealand white rabbits (Zhu *et al.,* unpublished data). The laboratory-obtained larvae and nymphs were from one of these females. These facts clearly show an almost 100 % transstadial and transovarial transmission of RMs *via* female gonads; while transovarial transmission of *E. phagocytophila* appears not to occur (Macleod and Gordon, 1933; Macleod, 1936; Foggie, 1951; Lewis, 1977, 1979; Woldehiwet, 1983). Thus, despite the morphological resemblance, it cannot be assumed that the RMs detected within the

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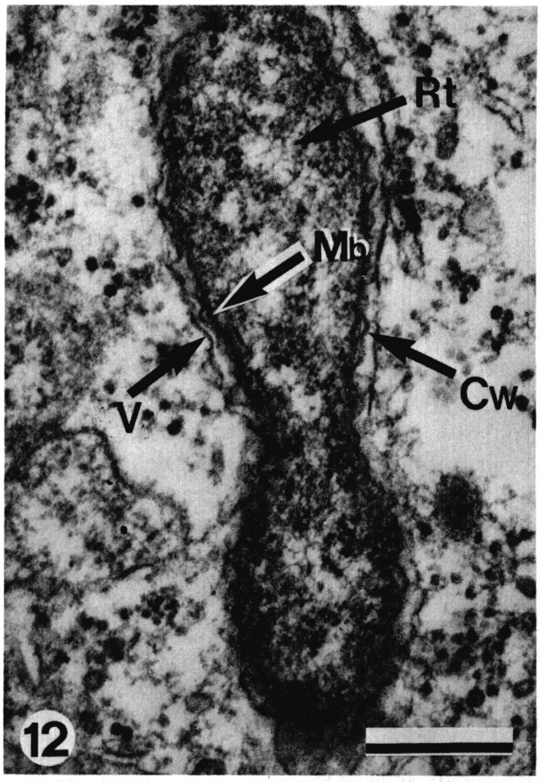
FIG. 12. — Profile of a RM within the ovarian tissue of a nymph at day 21 after repletion. Note the reticulated cytoplasmic matrix (Rt), the highly rippled outer cell wall (Cw) and the inner plasma membrane (Mb) of the microorganism, and the enclosing membrane-limited vacuole (V). Electron micrograph. Bar = 0.25 μm.

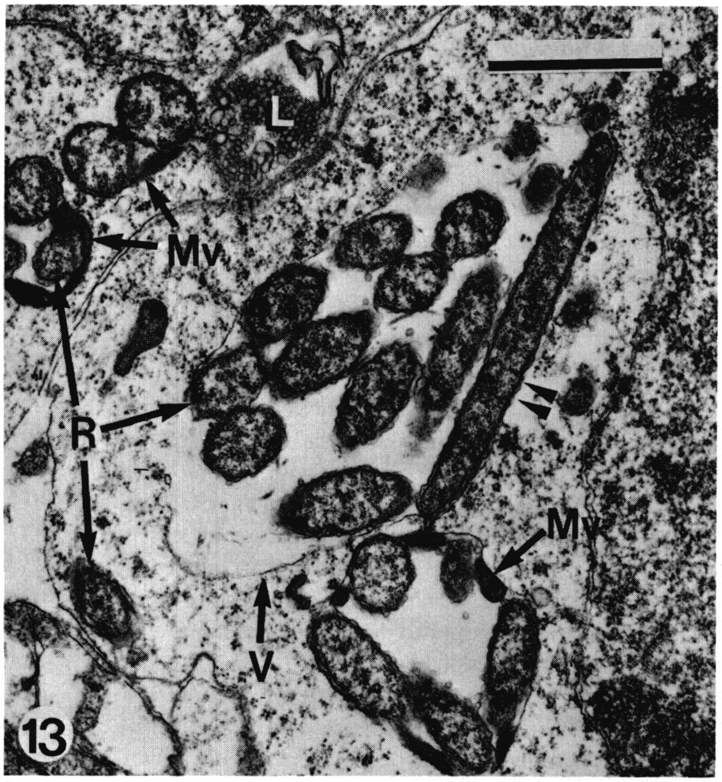
FIG. 13. — Ultra-thin section of the ovarian tissue from a nymph at day 21 after repletion, showing grouped RMs (= R) in a membrane-limited vacuole (V) and in the MMLVs (= Mv). Note an extremely long microorganism (approximately 2.5 μm long) (double small arrowheads), in the membrane-limited vacuole. L lysosome-like multi-vesicular vacuole. Electron micrograph. Bar = 1 μm.

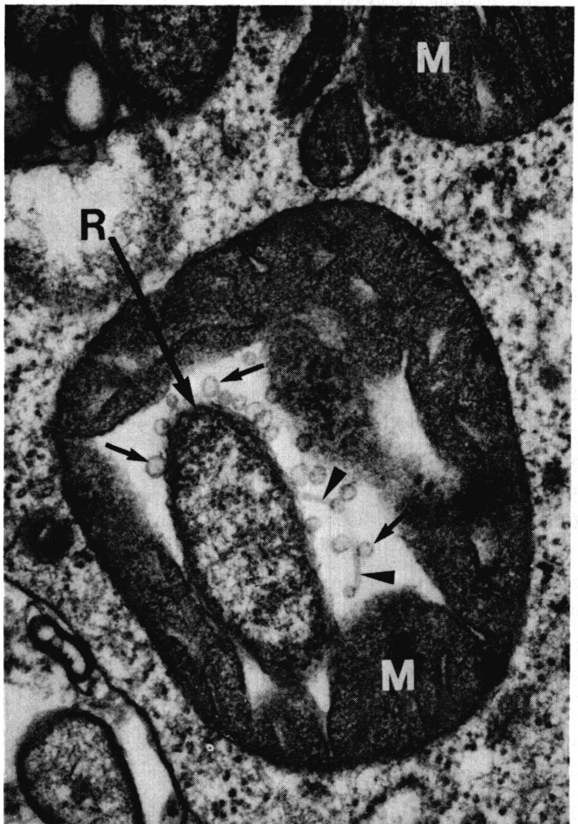
FIG. 14. — Ultra-thin section of the ovarian tissue from a nymph at day 15 after repletion, displaying two RMs (= R), one of which is not evident, within a vacuolated mitochondrion (M). Note many small rounded vesicles (small arrows) and a fine long tubule (arrowheads), with an even width and an arched appearance, associated with the RMs within the mitochondrion. Most of the small vesicles are still in contact with the rippled outer cell walls of the microorganisms. Several small vesicles are adhering to the fine tubule. Electron micrograph. Bar = 0.3 μm.

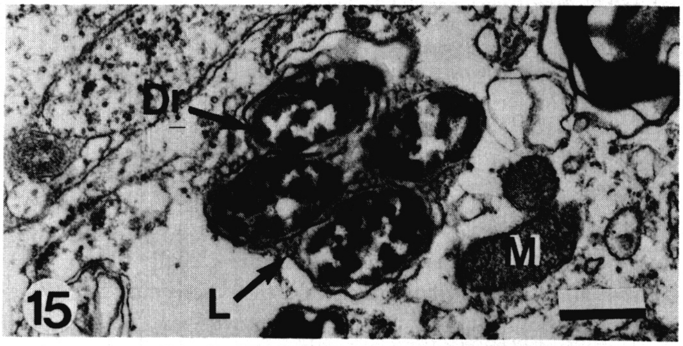
FIG. 15. — Degenerating RMs (= Dr) in a lysosome-like multi-vesicular vacuole (L) in a degenerating ovarian cell of a nymph at day 21 after repletion. Note the large electron-dense areas in the cytoplasmic matrix of the microorganisms and the small lysosome-like vesicles within the vacuoles. M mitochondrion. Electron micrograph. Bar = 0.5 μm.

FIG. 16. — Degenerating RMs (= Dr) in the lysosome-like multi-vesicular vacuoles (L) within a degenerating ovarian cell of a nymph at day 21 after repletion. Note that each degenerating microorganism has one large electron-dense area and many filamentary structures in the electron-transparent cytoplasmic matrix. Electron micrograph. Bar = 0.5 μm.









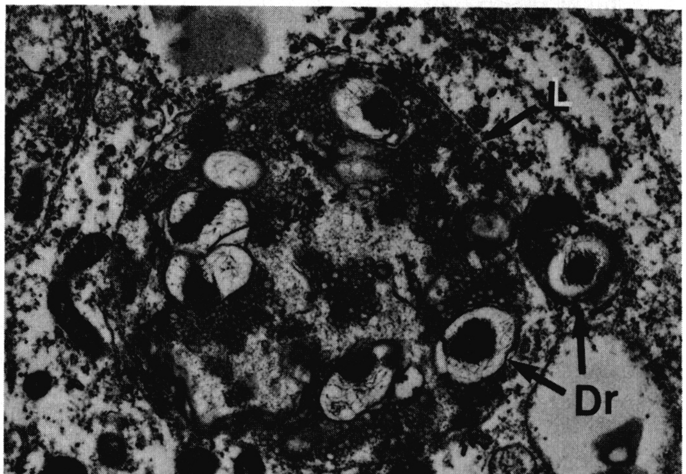


PLANCHE IV.

female gonads of *I. ricinus* from Neuchâtel, Switzerland are *E. phagocytophila,* the causative agent of tick borne fever, a cattle disease occurring also in Switzerland, but only in some isolated areas, mainly in the regions Vaud-Valais and Southern Berne (Pfister *et al.,* 1987; Liz *et al.,* 1991). This disease has never been reported in the region where the ticks were collected (Jorge Liz, personal communication). Whether the female gonads of *I. ricinus* from Neuchâtel harbour both transovarially transmissible ehrlichia-like rickettsiae appearing usually in groups within vacuoles, and symbiotic rickettsiae occuring singly or in groups within vacuoles, remains to be investigated.

The absence of the RMs in the male gonads of immature *I. ricinus* is in accordance with the findings for the RMs in *R. sanguineus, Boophilus annulatus, Dermacentor reticulatus* (Mudrow, 1932) and *A. persicus* (Roshdy, 1964*a*). However, the present electron microscope observations failed to detect RMs in the malpighian tubules of the immature stages of *I. ricinus.* In addition, neither ultrathin sections nor silver-stained histological sections had revealed RMs in the Malpighian tubules of bloodfeeding nymphs and females of *I. ricinus* (Zhu *et al.,* unpublished data). [Dieterle silver stain used for the detection of spirochetes (Van Oden and Greer, 1977; Gern, *et al.,* 1990; Zhu *et al.,* 1990) was proved to be also a good technique for the coloration of the present RMs and the Swiss agent (« *Rickettsia helvetica* ») discovered in *I. ricinus* in Switzerland (Aeschlimann *et al.,* 1979; Burgdorfer *et al.,* 1979; Hayes *et al.,* 1980; Péter *et al.,* 1981), and were employed for the histological localization of these two microorganisms in *I. ricinus* in our laboratory (Zhu *et al.,* unpublished data)]. These facts suggest that the present RMs may be transmitted transovarially and transstadially only *via* the female gonads of the tick, and that there may be no cross-infection (Mudrow, 1932) between ovary and other organs in all three stages of female ticks.

The present investigation shows that the distribution of the RMs in the primordial ovaries of molting larval *I. ricinus* is similar to that described for the RMs (*Wolbachia persica*) in larvae of *A. persicus* (Roshdy, 1964*a*), *R. appendiculatus, R. sanguineus* and *B. annulatus* (Mudrow, 1932), and that the distribution of the RMs in the primordial ovaries of molting nymphs of *I. ricinus* at day 15 and 21 after repletion resembles that reported for the RMs in the first nymphs of *A. persicus* (Roshdy, 1964*a*) and in the nymphs of *R. appendiculatus, R. sanguineus* and *B. annulatus* (Mudrow, 1932). That is to say, RMs are, singly or in groups, diffusely located in the cytoplasm of the primordial ovarian cells in the immature stages of all these ticks.

The average lifetime of mitochondria is short, the renewal of the organelles may involve a process resembling the duplication of bacteria by fission, and the cell division can reduce the number of mitochondria in a filial cell (Geneser, 1986). Our results show that, despite a rapid multiplication of the ovarian cells in nymphs found during the period between day 15 and 21 (see above; Till, 1961), the density of mitochondria seems not to decrease *(Figs. 1-2, 6, 8).* This implicates a rapid duplication of mitochondria in the developing nymphal ovaries. In addition, we observed that in the ovarian primordia of the female nymphs examined at day 15 after repletion, only a small part of the RMs were contained within vacuolated mitochondria or MMLVs *(Fig. 6),* while in the nymphs examined at day 21 after repletion, the majority of the RMs were enclosed within MMLVs or within vacuolated mitochondria. Furthermore, the mitochondrion-associated groups of the RMs are obviously larger in the nymphs examined at day 21 after repletion than those in the nymphs at day 15 after repletion *(Fig. 8, 13).* Thus, the above phenomena not only implicate an increased multiplication of the RMs (Roshdy, 1964*a*), but also suggest that these RMs become more and more dependent on the mitochondria with the development of the female gonads during nymphal molting. Adenosine triphosphate (ATP) or other metabolites of mitochondria may be advantageous to the multiplication of the mitochondrion-associated RMs (Wright and Barr, 1980). Whether the RMs are able to enter the mitochondria, wether the rapid mulplication of the entered organisms can result in the burst of the infected mitochondria (Lewis, 1979) or whether the mitochondria are able to elongate themselves and enclose the nearby RMs needs further investigations. In addition, why do many mitochondria found within the ovarian tissue display a vacuolated appearance? What happens when an infected mitochondrion duplicates itself? Can the mitochondrion-associated microorganisms be inherited by the new generation of the organelle? At present, we can not answer these questions and the relationship between RMs and mitochondria remains still unclear.

The degenerating forms of the present RMs are mainly found in the degenerating ovarian cells. This phenomenon is in agreement with the observation on *W. pipientis* in *Culex pipiens* and on wolbachiae in the *Aedes scutellaris* group (Wright and Barr, 1980). The facts that only the female gonads of the immature *I. ricinus,* which harbour a large quantity of RMs, possess a great number of lysosome-like multi-vesicular vacuoles, and that the degenerating RMs are mostly found within such a lysosome-like multi-vesicular vacuole, strongly suggest a role of these lysosome-like multi-vesicular vacuoles in controling the excessive growth of RMs, that may cause death of their host (Burgdorfer *et al.,* 1973; Šuťáková and Řeháček, 1991).

The numerous large and irregularly shaped vesicles associated with grouped RMs within the membrane-limited vacuoles of host cells, and the numerous small and regularly rounded vesicles and long fine tubules within vacuolated mitochondria and MMLVs harbouring RMs have not yet been reported to be associated with any other RMs before. The origin of the large irregularly shaped vesicles in the membrane-limited vacuoles is unknown. Nevertheless, the presence of a greatly rippled or blebbeb outer cell wall of the RMs in the vacuolated mitochondria and MMLVs and the adhesion of many small rounded vesicles to this cell wall *(Figs. 4, 13-14)* suggest that these vesicles may derive from the blebbed outer cell wall of the RMs. The fine tubules within the RM-infected mitochondria and MMVLs have a long, even, undulate appearance, and their diameter is obviously less than that of the small rounded vesicles. Thus, it appears that they may not result from the rippled outer cell wall of the RMs. On the other hand, we could not observed their real conjunction with the enclosing mitochondria, though the contact of the fine tubules with mitochondria has been found. In addition, the inner membrane projections of mitochondria forming tubular « cristae » have only been found in some steroid hormone producing cells of mammalians (Krstic, 1976). Therefore, at present, the mitochondrion origin of these long fine tubules can unlikely be suggested. Further investigation on the rickettsia-like microorganism-host cell interactions will provide clues to the formation and function of these interesting structures.

The occurrence of the concurrent infection of RMs and *B. burgdorferi* within the ovarian tissue of molting nymphs is very interesting. The same phenomenon is also found in the ovaries of female *I. ricinus* (Zhu *et al.*, unpublished data).

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