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MORPHOLOGY AND DEVELOPMENT OF *COWDRIA RUMINANTIUM* IN *AMBLYOMMA* TICKS

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ABSTRACT

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The morphology and development of *Cowdria ruminantium* have been studied in *Amblyomma hebraeum* and *A. variegatum*. Colonies of *C. ruminantium* have so far been demonstrated microscopically in gut, salivary gland cells, haemocytes and malphighian tubules of infected *Amblyomma* ticks. Colonies in gut cells were seen in both unfed and feeding ticks but colonies in salivary gland acini were observed only in nymphs that had fed for 4 days. Although the predominant type seen in both tick stages was the reticulated form that appeared to divide by binary fission, electron dense forms were also present. The latter are similar to those forms documented in endothelial cells of the vertebrate host as well as in cell culture.

The presence of colonies of *C. ruminantium* in salivary glands of feeding ticks, along with the demonstration of different morphologic forms of the organism, suggests that a developmental cycle of the organism occurs in its invertebrate host. It is thought that organisms first infect and develop within gut cells. From there subsequent stages continue their development in haemolymph and salivary glands and are then transferred to the vertebrate host during tick feeding. Further studies are needed to completely understand the development of *C. ruminantium* in ticks and its subsequent transmission by these parasites.

INTRODUCTION

Cowdria ruminantium is currently classified as one of the rickettsiae in a large group of vector-borne parasites that can survive and grow only within living cells (Weiss & Moulder, 1984). Tick-borne rickettsiae can be grouped into 2 broad categories: (1) those organisms that live and multiply freely within the cytoplasm of host cells, and (2) those that occur only in membrane-bound inclusions within host cell cytoplasm. Organisms in the first category that live freely in the cytoplasm of cells include species of the genera *Rickettsia* and *Wolbachia*. Those in the second category, found only within a membrane-bound inclusion, include *Cowdria* as well as *Anaplasma, Ehrlichia* and *Coxiella*.

The development of rickettsial organisms in ticks has not been as well documented as has that of the protozoa, *Babesia* and *Theileria*. With the protozoan parasites, initial infection of ticks occurs in gut cells; a developmental cycle follows that ends in formation of the infective state transmitted via the salivary glands during tick feeding (Mehlhorn & Schein, 1984). Previously, much research on rickettsial organisms in ticks involved the type organism for the Order Rickettsiales, *Rickettsia rickettsii* (Burgdorfer & Varma, 1968). This organism has been reported to divide only by binary fission and to infect all tick tissues; the infection it produces has been referred to as "generalized", and its growth does not appear to involve a specific sequence of development. Transmission of *R. rickettsii* from one generation of acarines to the next occurs consistently via the ovary. In contrast, data reported on other rickettsiae [*Ehrlichia* (Nyindo, Ristic, Huxoll & Smith, 1971); *Cowdria* (Pienaar, 1970; Prozesky, Bezuidenhout & Paterson, 1986); *Anaplasma* (Kocan, 1986), and *Coxiella* (Baca & Paretsky, 1983)] suggest that more than one stage of these organisms occurs in the vertebrate host, cell culture and ticks. These stages may be part of a developmental sequence and involve reproduction by some means in additional to division by binary fission. Recent research on these inclusion-forming organisms in ticks suggests that their developmental cycles are more complex than was once thought.

Our understanding of the development of *C. ruminantium* in ticks has been advanced in recent years by use of the electron microscope, which allows differentiation of the organisms from inclusions in tick gut and salivary gland cells. Information regarding the demonstration and morphology of this parasite in its invertebrate host is discussed herein.

DEMONSTRATION OF *C. RUMINANTIUM* IN *AMBLYOMMA* TICKS

Microscopic studies

Cowdry's original description of the development of *Rickettsia ruminantium* in both the vertebrate and invertebrate hosts has provided the basis for further studies on this organism (Cowdry, 1925a; 1925b). The organisms were first described in the invertebrate host within sections of gut cells of nymphal and adult *Amblyomma hebraeum* that were exposed to *C. ruminantium* as larvae. The parasite was easily differentiated from symbiotic rickettsiae that were present in tissues of both infected and control ticks because the *Cowdria* organisms were "densely packed together in very characteristic clumps which were usually spherical. . ." and were unique when compared with that of other rickettsiae in ticks (*R. rickettsia, R. prowazeki* and *R. quintana*). The *Cowdria* organisms in ticks were pleomorphic, gram negative and stained either blue or red with Giemsa stain. Colonies of *Cowdria* were also demonstrated in paraffin sections of nymphs with Mallory's phloxine-methylene blue stain (Yunker, Kocan, Norval & Burridge, 1987). Giemsa, methyl green-pyronin Y and acridine orange stains gave unsatisfactory results in frozen sections prepared from ticks known to be infected (Bezuidenhout, 1984).

Recent studies have confirmed Cowdry's earlier findings, demonstrating with both light and electron microscopy that *C. ruminantium* occurs within membrane-bound colonies in gut epithelial cells of *Amblyomma* ticks. Colonies of *C. ruminantium* in gut cells of *A. hebraeum* were seen in adults infected as larvae (Bezuidenhout, 1984) and nymphs infected as larvae (Kocan, Bezuidenhout & Hart, 1987) and in *A. variegatum* adults infected as nymphs (Kocan, Morzaria, Voigt, Kiarie & Irvin, 1986).

In addition to the well described gut stages, colonies of *C. ruminantium* have occasionally been found in Giemsa stained haemocytes (Du Plessis, 1985). During light and electron microscopic studies they have also been seen in malpighian tubules (J. D. Bezuidenhout, unpublished results, 1985) and salivary glands of *A. hebraeum* nymphs (Kocan *et al.*, 1987).

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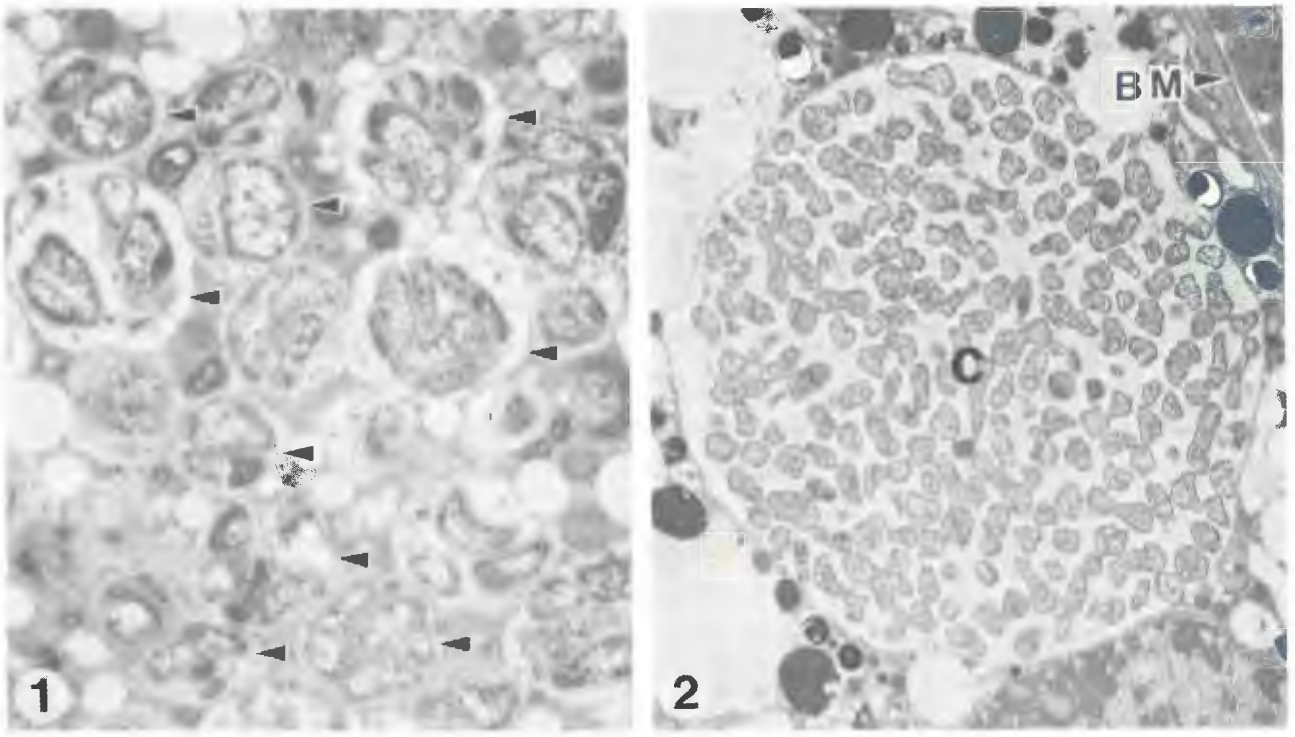

FIG. 1–2 Electron micrographs of *C. ruminantium* in a midgut epithelial cell of *A. variegatum* infected as nymphs. (1) Small colonies (arrows), first present at 15 days after tick repletion, contain organisms within a limiting membrane, × 12 000. (2) A large colony (C) of *C. ruminantium* near the basement membrane (BM) of a midgut epithelial cell from an adult *A. variegatum*, × 5 000.

Animal inoculation studies

Organ suspensions prepared from prefed *A. hebraeum* females infected as larvae with the Ball 3 strain were injected intravenously into sheep (J. D. Bezuidenhout, J. L. du Plessis & J. A. Olivier, unpublished data, 1985). Except for the suspensions made from the central nervous ganglion, haemolymph and ovaries, all the other organs (gut, salivary glands, hypodermis, malpighian tubes and rectal ampule) were infective. An interesting finding made during similar experiments with Kümm infected ticks was that gut suspensions were always uninfective while suspensions from the central nervous ganglion were always highly infective. The significance of these differences is not understood at present.

Saliva collected from engorged *A. hebraeum* females has also been infective in certain instances (Bezuidenhout, 1981).

Serological methods

Both direct and indirect fluorescent antibody techniques were applied on whole adult ticks to demonstrate specific staining of colonies of *C. ruminantium* in gut epithelium cells. These colonies corresponded exactly with those viewed under normal white light after counter staining with Evans blue (Bezuidenhout, 1984).

Recently the enzyme-linked immunosorbent assay (ELISA) technique has been successfully used for the identification of *C. ruminantium* in ticks (Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen, 1986). It has also been used to determine the presence of organisms in tick organ suspensions (G. J. Viljoen, J. D. Bezuidenhout, A. W. H. Neitz & N. M. J. Vermeulen, unpublished data, 1986).

MORPHOLOGY AND DEVELOPMENT OF *C. RUMINANTIUM* IN *AMBLYOMMA* TICKS

Morphology of gut stages

Light microscopy.—Colonies of *C. ruminantium* were present in 1 μm plastic sections of midgut epithelial cells of both nymphal and adult *A. hebraeum* infected as larvae and in adult *A. variegatum* infected as nymphs. Colonies varied greatly in size: 3–45 μm in *A. hebraeum* (Bezuidenhout, 1984), and 1–60 μm in *A. variegatum* (Kocan *et al.*, 1986).

A. variegatum, infected with the Kiswani isolate of *C. ruminantium*, appeared to have much higher numbers of colonies in gut cells than did *A. hebraeum* infected with the Ball 3 strain.

Electron microscopy.—Apparently colonies of *C. ruminantium* did not start to form until after the ticks had fed to repletion on an infected animal. In *A. variegatum*, colonies were not present in ticks collected while feeding on an infected animal, but small colonies became apparent in gut cells of nymphs 15 days after the ticks had fed to repletion (Fig. 1 & 2). The organisms were always within an inclusion membrane, even in small colonies containing only 1 or 2 organisms (Fig. 1) (Kocan *et al.*, 1986). The predominant stage of *C. ruminantium* within colonies in gut cells was the reticulated form (Fig. 2), which appeared to be dividing by binary fission. Electron-dense forms were also present in small newly-developed colonies in *A. variegatum* (Fig. 1) (Kocan *et al.*, 1986) and in gut cells of *A. hebraeum* that were infected as larvae (Fig. 3–6) (Kocan *et al.*, 1987). Electron-dense forms in *A. hebraeum* nymphs occasionally contained a crystalline core (Fig. 6) (Kocan *et al.*, 1987). In both species of ticks, colonies often contained an electron-dense inclusion to which organisms were directly adhered (Fig. 4). The inclusion was similar in morphology and staining qualities to haemoglobin inclusions normally found in tick gut cells, suggesting a direct dependence of the organism upon this material (Kocan *et al.*, 1986; Kocan *et al.*, 1987).

Morphology of salivary gland stages

Light microscopy.—Colonies of *C. ruminantium* were observed in 1 μm plastic sections of salivary gland acini from *A. hebraeum* nymphs that were infected as larvae

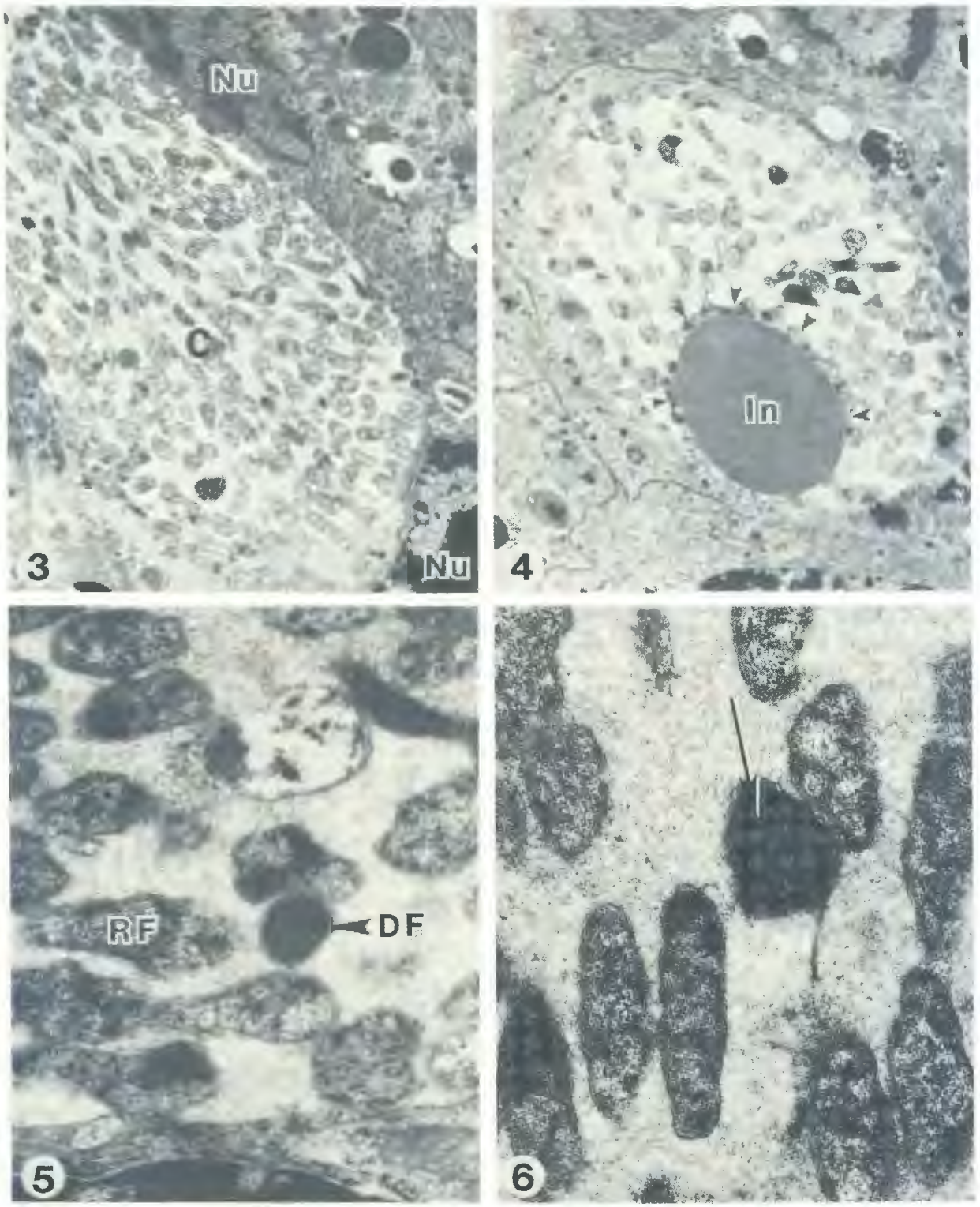

FIG. 3–6 Electron micrographs of *C. ruminantium* in midgut epithelial cells of *A. hebraeum* that fed as larvae on an infected sheep. (3) A colony (C) within a midgut epithelial cell adjacent to two host nuclei (Nu), × 5 000. (4) A colony (C) containing an electron-dense inclusion (In) with *C. ruminantium* organisms adhered to the surface (arrows), × 6 000. (5) A high magnification of reticulated (RF) and electron-dense (DF) forms of *C. ruminantium* within a gut colony, × 20 000. (6) An electron-dense form that contains a crystalline formation (arrow), × 20 000

and were allowed to feed for 4 days (Kocan *et al.*, 1987) (Fig. 7 & 8). The resolution of these sections allowed for the differentiation of colonies from the many inclusions and granules that normally occur in salivary gland cells. The diameter of the colonies ranged from 5 μm to 30 μm and the morphology varied from smaller, densely-staining colonies to larger ones in which individual organisms were clearly visible (Fig. 7 & 8). The colonies were only seen in simple granular cells of type II and III acini.

Electron microscopy.—Most colonies of *C. ruminantium* in salivary glands contained reticulated forms that appeared to be reproducing by binary fission (Fig. 9–12) and were similar morphologically to colonies of the organism demonstrated in gut cells. A few colonies contained electron-dense forms of the parasite (Fig. 10), but organisms within colonies often had an electron-dense core (Fig. 12). Smaller colonies were also densely-packed with reticulated organisms (Fig. 11). Within

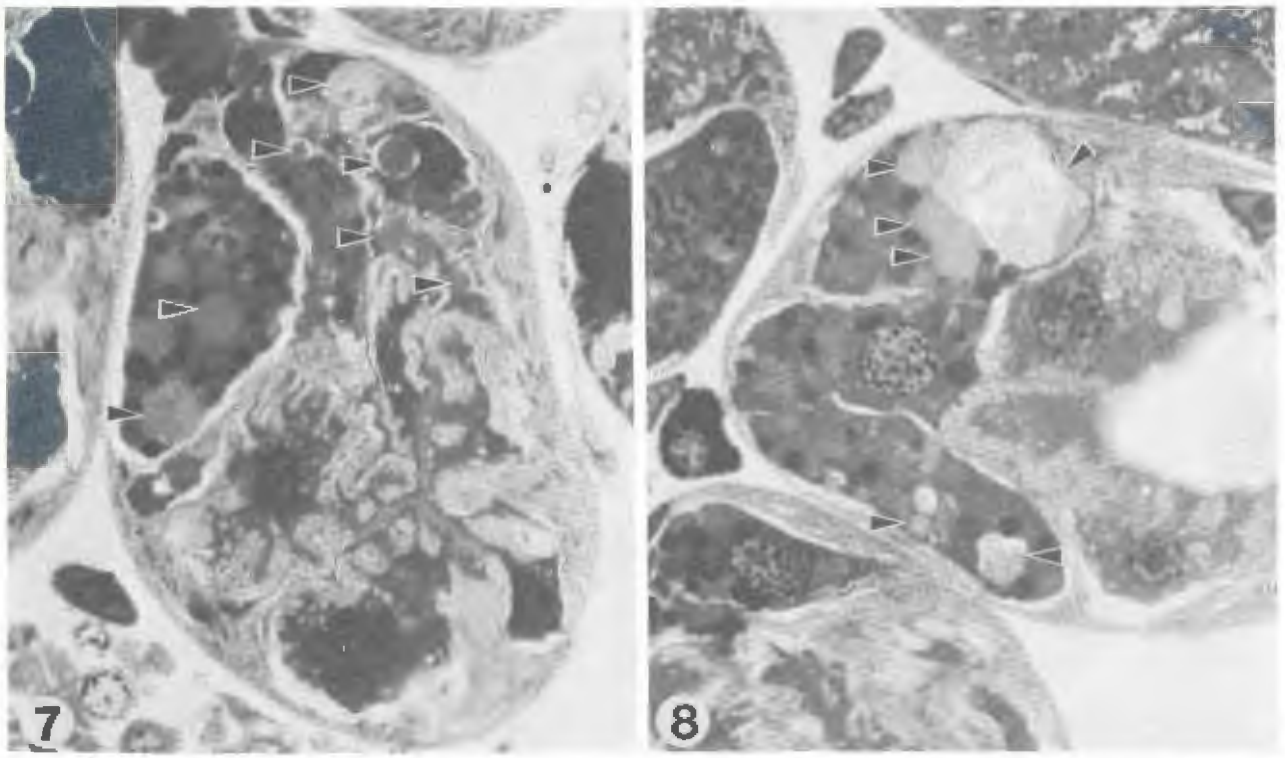

FIG. 7–8 Photomicrographs of *C. ruminantium* in cross sections of salivary gland acinus from nymphal *A. hebraeum* that were infected as larvae and had fed for 4 days. The 1 μm plastic sections are stained with Mallory's stain. Colonies vary in morphology from small, densely staining colonies to larger colonies with separated organisms (arrows), × 2 000

colonies in salivary glands and gut cells, a moderately-dense material was in close association with the organism (Fig. 12).

DISCUSSION

Recent studies have confirmed the findings of Cowdry (1925b) that *C. ruminantium* occurs in gut epithelial cells of *A. hebraeum,* and that similar colonies of *C. ruminantium* also occur in infected *A. variegatum.* Electron microscopy has further shown that the "clumps" or organisms that Cowdry described with light microscopy are actually colonies of organisms bounded by an inclusion membrane.

In both the vertebrate and invertebrate hosts, *Cowdria* is consistently found within an inclusion vacuole rather than free within the host cell cytoplasm. The occurrence of this organism in colonies distinguishes it from symbiotic rickettsiae that are commonly found in ticks but, unlike *Cowdria,* rarely form inclusions.

Cowdry hypothesized that transmission of the organism occurred via gut regurgitation during tick feeding, because clumps of organisms were seen in the lumen of the gut and he was unable to demonstrate similar clumps in salivary glands of ticks known to be infected. The recent demonstration of *C. ruminantium* in salivary glands of feeding, infected ticks suggests that the organism is transferred to the vertebrate host via this tissue rather than by gut regurgitation as suggested by Cowdry. Although the colonies in salivary glands have escaped recognition previously, their morphology is quite similar to that of colonies found in gut cells. Since salivary gland cells contain many inclusions it is easy to overlook the *Cowdria* colonies. The use of 1 μm plastic sections provides better resolution of cell morphology than thicker paraffin sections, thus allowing for differentiation of colonies from other normal cell components. Also, the colonies appear to develop within a specific salivary gland cell. Although relatively few (15 %) of the tick salivary glands studied were found to be infected, individual cells contained several colonies of organisms. The stages observed in salivary glands were found in nymphal ticks similar to those used for the heartwater tick derived vaccine. Fully engorged nymphae that were infected as larvae were highly infective; only 0,0015 of a homogenized nymph was necessary to cause infection in a susceptible sheep (Bezuidenhout, 1981). These data suggest that the stages in salivary glands may be highly infective.

With electron microscopy, different stages of *Cowdria* were seen within colonies in tick cells. These stages were similar to those described for the development of *Cowdria* in the vertebrate host (Pienaar, 1970) and more recently in cell culture (Prozesky *et al.,* 1986). Although the predominant form seen in gut and salivary gland colonies was reticulated, electron-dense forms were also present. These appear to be part of a developmental sequence of *Cowdria,* and are similar in some respects to stages demonstrated with *Anaplasma marginale* in ticks (Kocan, 1986) and with chlamydial organisms (Storz & Spears, 1977). The reticulated form appears to be the predominant vegetative stage within cells because of the morphologic evidence of binary fission. Infective resistant forms of *Cowdria* have not been identified. However, we can presume that the electron-dense forms of *Cowdria* may be similar to that of the elementary body (EB) organisms (Storz & Spears, 1977). Colonies of *Cowdria* in tick haemolymph (Du Plessis, 1985) may be intermediate forms in transit from gut to salivary gland cells.

In future, morphological studies on the organism in the tick should be aimed at demonstrating a definite life cycle. Furthermore, the multiplication of the various *Cowdria* isolates in the different *Amblyomma* vectors should be compared by using suitable techniques. This information could be very useful in the identification of suitable strains of *Cowdria* and ticks for the preparation of the highly infective suspensions needed for future studies.

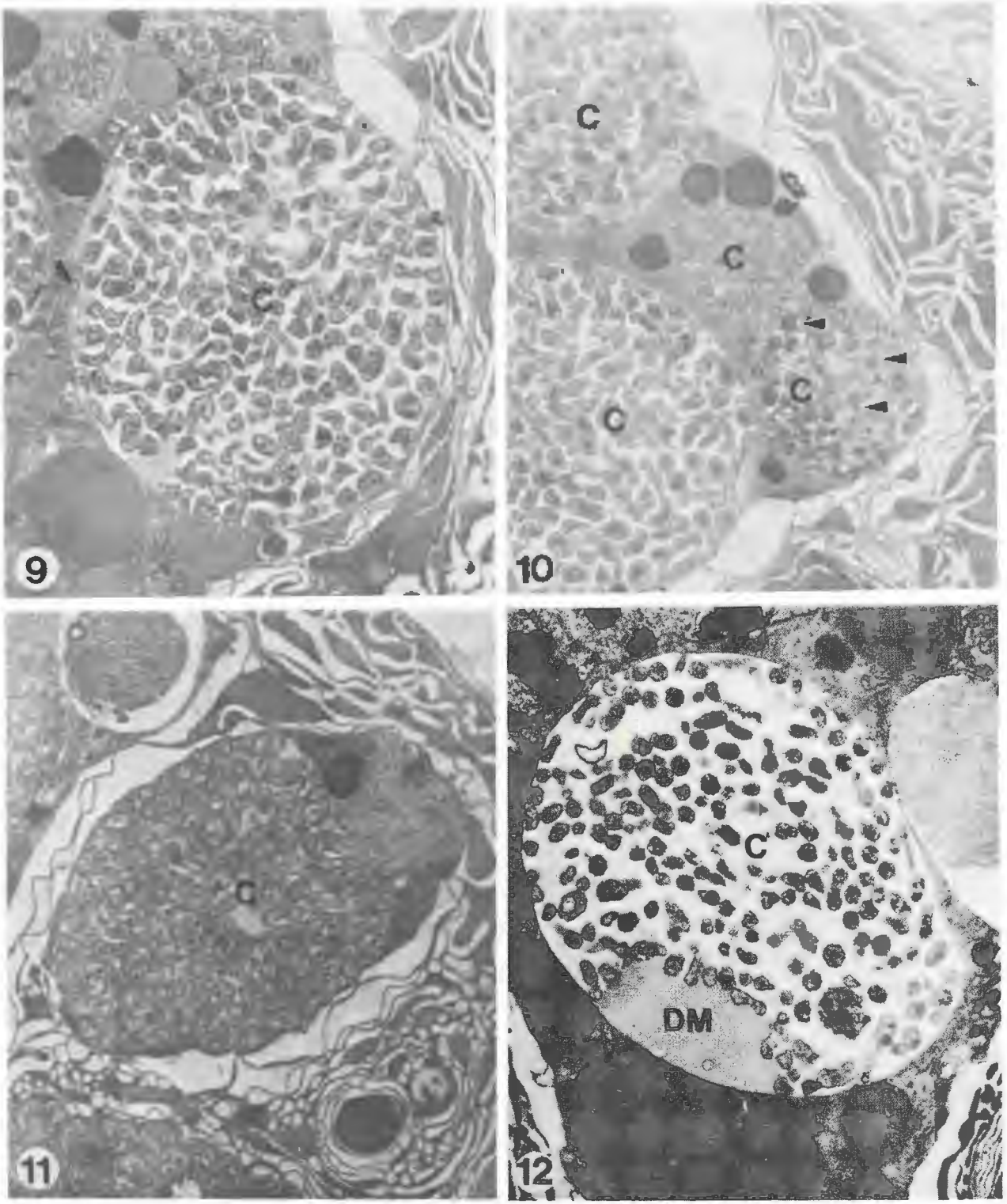

FIG. 9–12 Electron micrographs of *C. ruminantium* in salivary gland acinus of nymphal *A. hebraeum* that had fed for 4 days. (9) A large colony (C) within a simple granulated cell of a salivary gland acinus containing reticulated forms of the organism, × 6 000. (10) A salivary gland acinus cell with 4 colonies (C) of *C. ruminantium*. One colony appears to contain electron-dense forms (arrows) while the others contain reticulated forms of the parasite, × 6 000. (11) A colony (C) of *C. ruminantium* contains densely packed, reticulated organisms, × 6 000. (12) A colony containing moderately dense material (DM) with *C. ruminantium* organisms attached to its surface, × 5 000

A simple laboratory technique to demonstrate *Cowdria* in the tick is required in order to determine the infection rate of ticks and to study the factors that may influence such a rate.

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