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ULTRASTRUCTURAL MORPHOLOGY OF *COWDRIA RUMINANTIUM* IN MIDGUT EPITHELIAL CELLS OF ADULT *AMBLYOMMA HEBRAEUM* FEMALE TICKS

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ABSTRACT

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Amblyomma hebraeum male and female ticks, experimentally infected as larvae with the Ball 3 stock of *Cowdria ruminantium*, were fed on a heartwater susceptible sheep. The initial attachment of the males was required as a pre-requisite for female attachment.

Reticulate bodies were the predominant morphologic form of *Cowdria* observed in gut epithelial cells after 1–3 days of feeding. Single intermediate bodies and no elementary bodies were observed. Organisms were found within a membrane-bound vacuole and each organism had a double-unit membrane. Infrequently colonies contained homogeneous electron-dense inclusions. Groups of *Cowdria* organisms within a haemocyte suggested a possible dissemination of organisms from the gut to various other tissues by haemocytes.

INTRODUCTION

Cowdry (1925) identified *Cowdria ruminantium*, the causative agent of heartwater, in the gut epithelial cells and gut lumen of adult *Amblyomma hebraeum* ticks which had fed on sheep, goats and cattle inoculated intravenously (IV) with *C. ruminantium* infected blood. Subsequently various workers have studied the morphology, distribution and occasionally the life cycle of *C. ruminantium* in the vertebrate host (Pienaar, 1970; Du Plessis, 1975; Du Plessis, 1985; Prozesky & Du Plessis, 1985; Prozesky, 1987; Prozesky & Du Plessis, 1987; Logan, Whyard, Quintero & Mebus, 1987; Jongejan, 1990), in the tick vector (Bezuidenhout, 1984; Du Plessis, 1985; Kocan & Bezuidenhout, 1987; Kocan, Bezuidenhout & Hart, 1987a; Kocan, Morzaria, Voigt Kiarie & Irvin, 1987b; Yunker, Kocan, Norval & Burridge, 1987) and *in vitro* (Bezuidenhout, Paterson & Barnard, 1985; Prozesky, Bezuidenhout & Paterson, 1986; Logan *et al.*, 1987; Prozesky, 1987; Prozesky & Du Plessis, 1987; Jongejan, 1990).

Based on transmission electron microscopical observations, four different morphological forms of *Cowdria* were observed in endothelial cells in the choroid plexus of sheep infected with the Ball 3 stock i.e. small (0,49 µm), medium sized (0,76 µm), large (1,04 µm) and giant forms (2,7 µm) (Pienaar, 1970). Furthermore, the morphology of the Welgevonden stock of *Cowdria in vitro* was found to be very similar to the Ball 3 stock of *Cowdria* in the vertebrate host (Prozesky, 1987).

Du Plessis (1975) described the morphology of the Kümm stock of *Cowdria* in various cells of mice viz., peritoneal macrophages, Kupffer cells, suspected sinusoidal endothelial cells of the liver, myocardial capillary endothelial cells and splenic histiocytes. He also described the morphology of the Ball 3 stock in lymph node reticulo-endothelial cells of sheep and cattle. The morphology of developing organisms in the mouse peritoneal macrophages closely resembled those in the mouse Kupffer cells and lymph node reticulo-endothelial cells of ruminants. From these findings Du Plessis (1975) proposed a life cycle whereby an infective organism representing a very dense, finely granular body, infects the host cell (mouse peritoneal macrophages or reticulo-endothelial cells), increases in size to form fragmented dense bodies, and by further subdivision and organization appear to form colonies of organisms. This has not been confirmed by other workers.

C. ruminantium colonies were identified in the salivary gland acini epithelial cells in 4 out of 20 nymphae that had fed for 4 days on a heartwater susceptible sheep. These nymphae had been fed as larvae on an infected sheep (Kocan *et al.*, 1987a). *Cowdria* colonies were also observed in midgut epithelial cells in unfed infected *A. hebraeum* nymphae and nymphae that had fed for 1–4 days. No organisms, however, were observed in the midgut epithelial cells of nymphae that had fed for 5 days. Bezuidenhout (1984) identified *Cowdria* colonies in the gut epithelial cells of adult *A. hebraeum* ticks while Kocan *et al.* (1987b) found colonies of *C. ruminantium* in the gut epithelial cells of both nymphal and adult *A. variegatum*.

Cowdria colonies within the gut and salivary gland epithelial cells of *A. hebraeum* nymphae were morphologically similar to those found in the endothelial cells of the vertebrate host and in cultured endothelial cells. The different morphological forms may represent different stages in the life cycle of *C. ruminantium* (Kocan *et al.*, 1987a).

The method of transmission of *C. ruminantium* from the tick vector to the vertebrate host has as yet not been elucidated (Kocan *et al.*, 1987a). Initially it was thought that *C. ruminantium* was transmitted to the vertebrate host by means of regurgitation of the intestinal contents during feeding (Cowdry, 1925, 1926; Bezuidenhout, 1981; Uilenberg, 1983; Brown, 1988). Bezuidenhout (1981, 1988) studied the infectivity of the saliva and gut suspensions of engorged *A. hebraeum* adult females and nymphae, respectively, by injecting saliva and gut suspensions IV into susceptible sheep. The infectivity of saliva from the engorged adults was very low compared to that of the gut suspensions of the engorged nymphae. Van Winkelhoff (unpublished data, 1979, cited by Uilenberg, 1983) inoculated goats IV with salivary gland suspensions of infected unfed *Amblyomma variegatum* adults and infected *A. variegatum* adults that had fed for 2–3 days. None of the goats contracted heartwater. However, in other studies (Bezuidenhout, 1988) it was found that salivary gland homogenates from *A. hebraeum* females were constantly infective.

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The current paper reports on the development and morphology of *C. ruminantium* within the gut epithelial cells of adult *A. hebraeum* females. This will enable a comparison between the different morphological forms within the adult and nymphal ticks as well as those in tissue culture and the vertebrate host.

MATERIALS AND METHODS

Experimental procedure

One hundred *A. hebraeum* males and 100 females (Wessels stock), infected as larvae with the Ball 3 stock of *C. ruminantium* (Bezuidenhout, 1981) were fed in 2 calico bags on the back of a heartwater susceptible sheep according to the method of Heyne, Elliott & Bezuidenhout (1987). The males were allowed to feed 4 days before the females, as the presence of males is essential for the attachment of the females. The bags were checked daily for attachment of the ticks. Five females were removed on each day of feeding (Table 1). Control ticks consisted of the same number of *A. hebraeum* (Wessels stock) non-infected male and female ticks. They were fed and treated in the same manner on a second heartwater susceptible sheep. On each day of feeding 5 females were removed (Table 1).

TABLE 1 Feeding of *A. hebraeum* females on 2 heartwater susceptible sheep

Removal of ticks after feeding (Days)	Number of ticks removed
1	5
2	5
3	5
4	5
5	5
6	5
7	5
8	5
Replete (9 Days)	5

Collection of tissues

Various tissues, including the gut and salivary glands, were collected as outlined below, from infected female ticks that had fed for 1–8 days respectively and from replete females (Table 1). Tissues from the non-infected control ticks were processed in the same manner as described for the infected ticks.

After slicing the tick body with a sharp scalpel blade between the mouth parts and forelegs, the organs were gently removed from the tick body and fixed in cold (4 °C) 2 % glutaraldehyde in a 0,2 M sodium cacodylate buffer with 0,5 % sucrose. From day 7 onwards this method became unpractical and the organs had to be dissected from the ticks. Tissues were post-fixed in 2 % osmium tetroxide in 0,2 M sodium cacodylate buffer and processed for transmission electron microscopy according to the method of Kocan, Venable & Brock (1978).

Semi-thin sections were stained with Mallory's stain (Richardson, Jarret & Finke, 1960) for 2 min at 60 °C and examined with a light microscope for the presence of *C. ruminantium* colonies. From the selected blocks ultra-thin sections were collected on 200–300 mesh copper grids and stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1965).

RESULTS

Experimental animal

C. ruminantium was transmitted to the sheep on which adult male and female *A. hebraeum* ticks, infected as larvae with the Ball 3 stock, were fed. Twenty-seven days after exposure to the ticks the febrile reaction of the animal attained 41,5 °C. It appeared dull and showed intermittent nervous symptoms 28 days post infection. On that day the sheep was treated with 10 mℓ Curamycine LA* IV and 6 days later challenged with 5 mℓ *C. ruminantium* infective blood issued as a vaccine by the Veterinary Research Institute, Onderstepoort. The animal was found to be immune to the challenge. Control male and female ticks did not cause the other sheep to contract heartwater and upon challenge, the sheep was found to be susceptible to heartwater.

Light microscopy

Midgut epithelial cells: One to approximately 16 *C. ruminantium* colonies were detected in single gut epithelial cells of ticks that had fed for 1–3 days. Colonies varied in size from 4,0 µm–60,0 µm in diameter. The staining intensity of the matrix in which the *Cowdria* organisms were suspended, ranged from blue for the smaller colonies (<*c* 20,0 µm in diameter) to translucent for the larger colonies. Irrespective of the staining intensity of the matrix the individual organisms within a colony were always clearly visible (Fig. 1). No organisms were identified within the gut epithelial cells from days 4 to repletion and no organisms were identified in the gut epithelial cells of the control females that had fed from days 1 to repletion.

Salivary glands: Although numerous sections from salivary glands of females that had fed for 1 day to

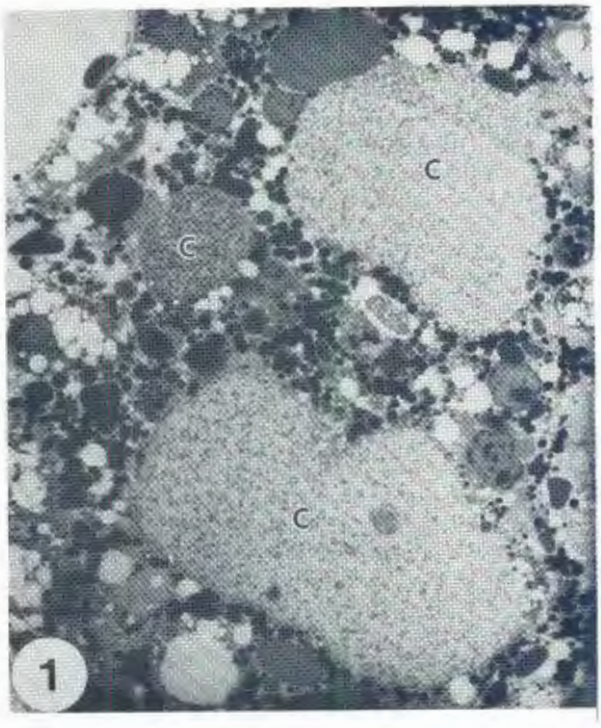

FIG. 1 *C. ruminantium* colonies (C) in gut epithelial cells of female *A. hebraeum* ticks: × 1000

* Oxytetracycline. Agricura (Pty) Ltd, Republic of South Africa

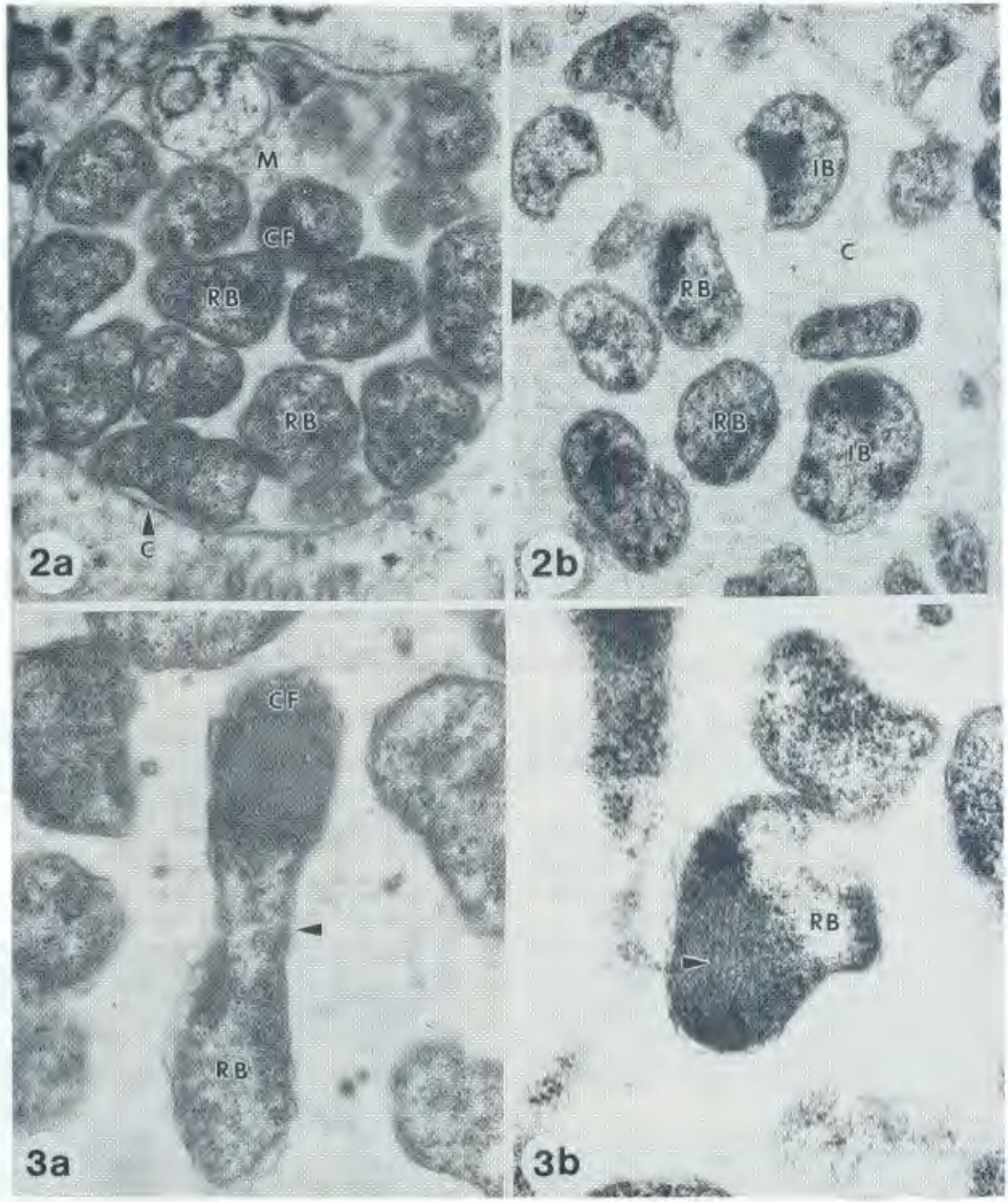

FIG. 2a *C. ruminantium* colony (arrow) containing reticulate bodies (RB) within a medium electron-dense colony matrix (M). A crystalline form is evident in an organism (CF): × 6000

FIG. 2b Reticulate bodies (RB) and intermediate bodies (IB) within a colony (C): × 5000

FIG. 3a Reticulate body undergoing binary fission (arrow) forming a reticulate body (RB) and an organism with a crystalline form (CF) in transverse view: × 40000

FIG. 3b Reticulate body (RB) with a crystalline form, illustrating the tubular character (arrow): × 40000

repletion were examined, no *Cowdria* colonies were identified in neither the infected nor the control ticks.

Transmission electron microscopy

Midgut epithelial cells: The number, pattern of distribution and size of the colonies in gut epithelial cells in the same tick varied significantly. Although most of the colonies were round, the asymetrical colonies made exact measurement of the diameter difficult. Colonies measured 0,96 µm–48,0 µm in diameter.

Organisms were suspended in a matrix composed of fine fibrils within a membrane-bound vacuole. Often the fibrils were impregnated with small electron-dense, dust-like granules which gave the matrix a medium electron-dense appearance (Fig. 2a). The number of organisms in colonies studied on ultrathin sections, ranged from 4 to *c* 1100. Organisms

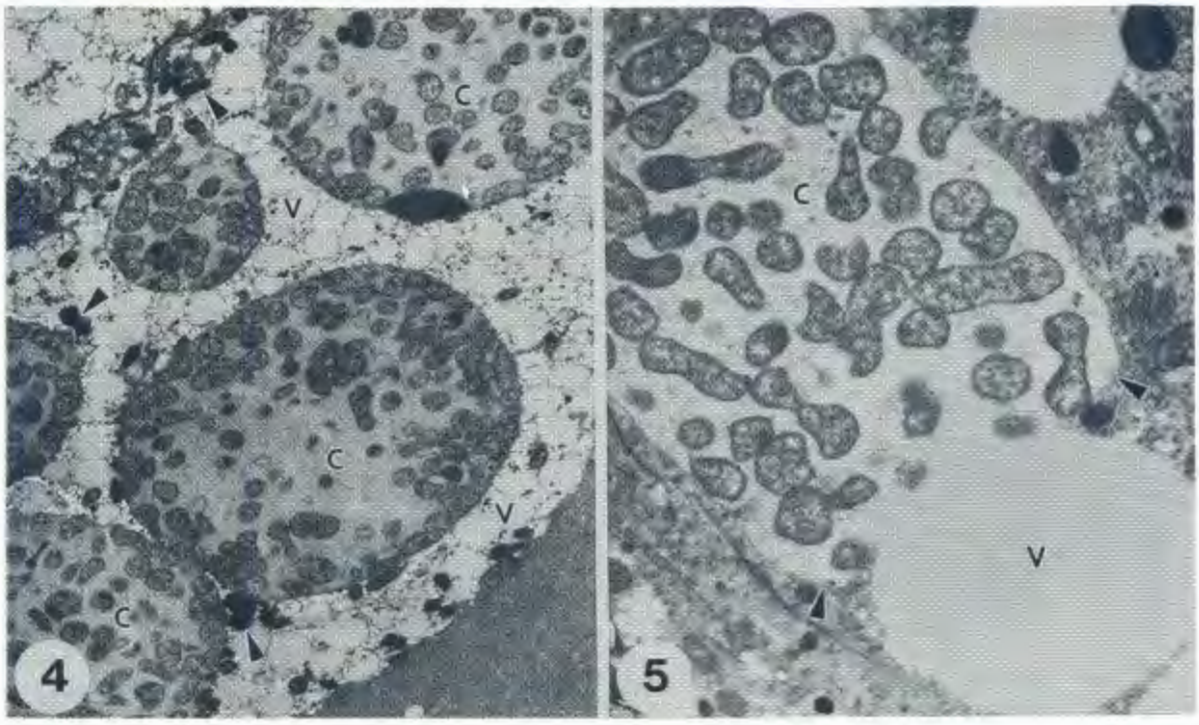

FIG. 4 *C. ruminantium* colonies (C) in midgut epithelial cells surrounded by membrane-bound vacuoles (V) and cytoplasmic inclusions (arrows): × 3000

FIG. 5 Large vacuole (V) which appears to merge with the surrounding membrane (arrows) of a colony (C): × 8000

within a colony were pleomorphic (0,34 μm–1,96 μm in diameter) and had a double membrane separated by a clear space. The outer membrane was often rippled.

The internal structure of reticulate bodies consisted of electron-dense and electron-pale areas (Fig. 2b). Single intermediate bodies with an electron-dense centre were observed (Fig. 2b). No elementary bodies (electron-dense bodies) were seen.

Crystalline forms showing varied patterns were sometimes observed within reticulate bodies. An organism undergoing binary fission forming a reticulate body and an organism with a crystalline form, apparently in a transverse view, is shown in Fig. 3a. A reticulate body with the crystalline form in a longitudinal view illustrating its tubular character, is shown in Fig. 3b.

The majority of *C. ruminantium* colonies were surrounded by membrane-bound vacuoles (*c* 0,41 μm–5,99 μm in diameter) and cytoplasmic inclusions (Fig. 4). Deformity of some colonies were caused by the surrounding vacuoles. Sometimes the larger vacuoles appeared to merge with the membranes surrounding a colony (Fig. 5). Electron-dense homogeneous inclusions (2,33 μm–3,63 μm in diameter) were occasionally seen in colonies on days 1 and 2 of feeding (Fig. 6).

A haemocyte containing 3 groups of *Cowdria* organisms and many inclusion bodies was noted in a *Cowdria* colony on day 2 of feeding (Fig. 7a). In one of these groups an organism was clearly surrounded by a double membrane (Fig. 7b). *Cowdria* organisms within the colony were in close proximity to the periphery of the haemocyte and were approximately of the same size as the organisms within the haemocyte (Fig. 7b). On days 1 and 2 of feeding single free organisms, were observed in the cytoplasm of midgut epithelial cells (Fig. 8).

On day 2 of feeding, groups of organisms (1,25 μm–13,90 μm in diameter) were seen at the periphery of a large colony and others were scattered further away in the cell cytoplasm (Fig. 9). Although some of these groups lay within a membrane-bound vacuole others were devoid of a membrane.

DISCUSSION

C. ruminantium colonies were found within the gut epithelial cells of adult *A. hebraeum* females dur-

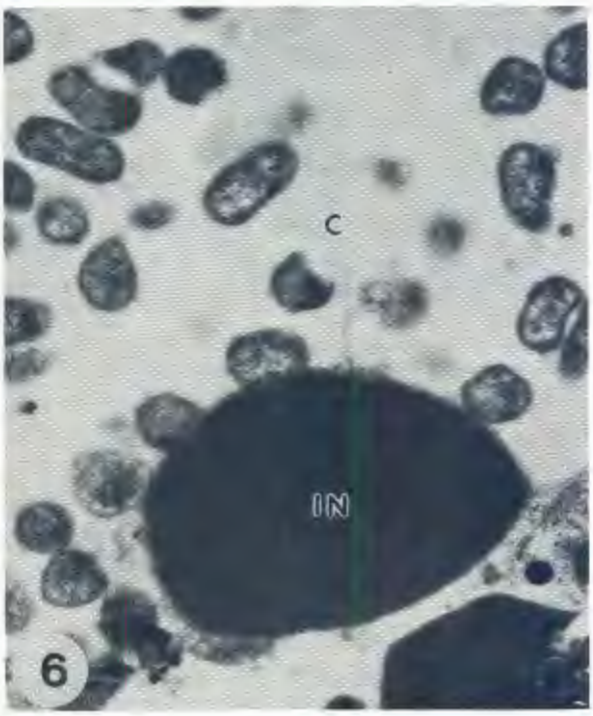

FIG. 6 Homogeneous electron-dense inclusion (IN) seen on days 1 and 2 of feeding within a *Cowdria* colony (C): × 8000

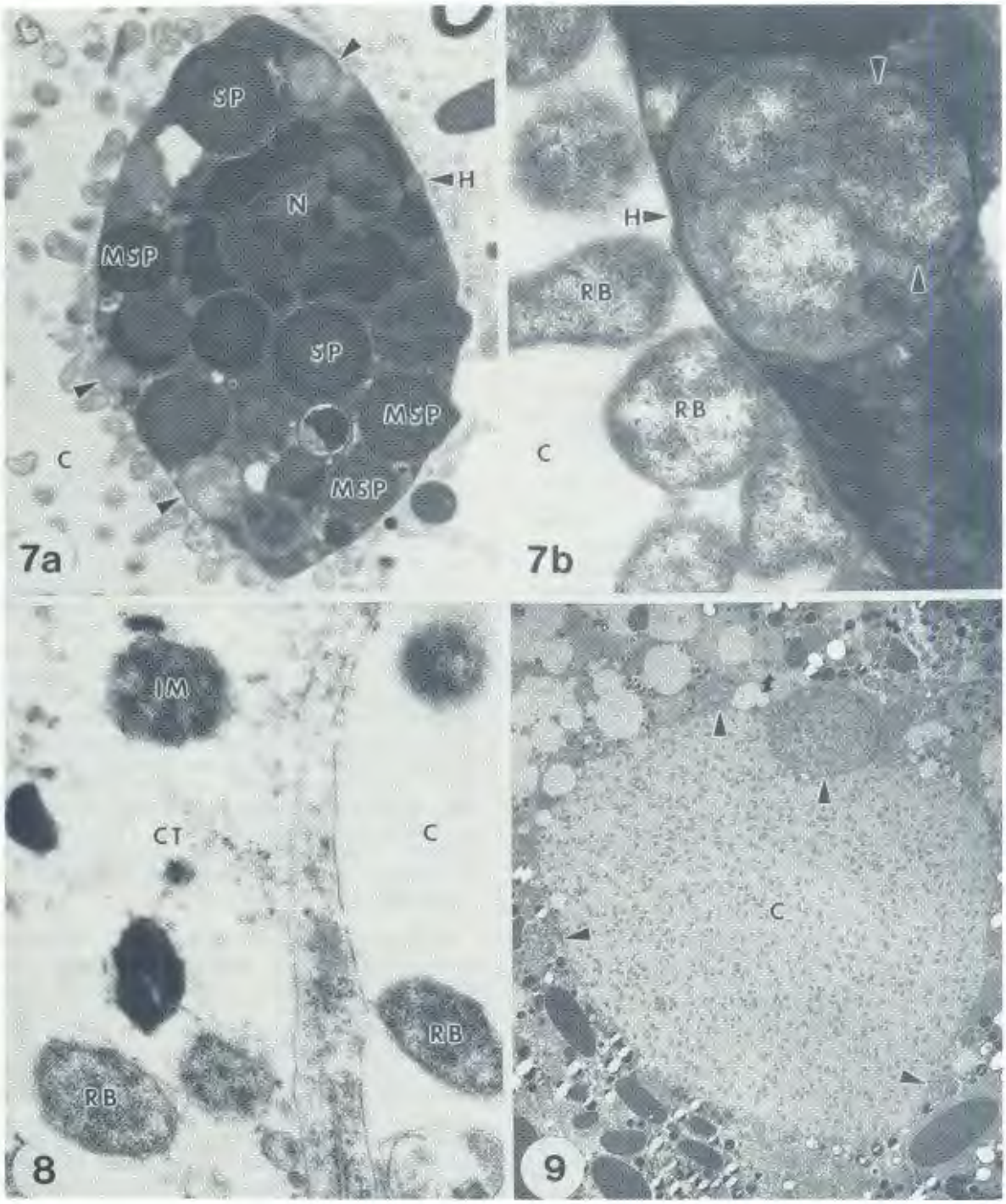

FIG. 7a Three groups of *Cowdria* organisms (arrows) and many spherules (SP) within a haemocyte (H) present in a *Cowdria* colony (C). Spherules with mottled appearance (MSP), nucleus (N): × 5000

FIG. 7b Higher magnification of a group of *C. ruminantium* organisms within the haemocyte (H). One of the organisms is clearly surrounded by a double membrane (arrows). Individual reticulate bodies (RB) within the colony (C) are in close proximity to the periphery of the haemocyte: × 30000

FIG. 8 Single free reticulate bodies (RB) and an intermediate body (IM) in the cytoplasm (CT) of a midgut epithelial cell adjacent to a *Cowdria* colony (C): × 15000

FIG. 9 A large *C. ruminantium* colony (C) in a midgut epithelial cell on day 2 of feeding. Groups of *Cowdria* organisms are situated at the periphery of the colony (large arrows) and groups of organisms are scattered throughout the cell cytoplasm (small arrows): × 1200

ing the first 3 days of feeding. This is in agreement with the results of Kocan *et al.* (1987a) who fed *A. hebraeum* nymphae for 5 days on a sheep infected with the Ball 3 stock of *C. ruminantium* and found *Cowdria* colonies in the gut epithelial cells on the first 4 days of feeding. However, no *Cowdria* colonies (Kiswani stock) were detected in the gut epithelial cells of *A. variegatum* nymphae fed for 6 days on heartwater infected cattle (Kocan *et al.*, 1987b). Conversely, organisms were observed in the gut epithelial cells of the nymphae 15 days post-repletion (Kocan *et al.*, 1987b).

According to Raikhel (1983) ixodid ticks ingest a small blood volume 1–3 days after attachment. This

is followed by a rapid ingestion of a large bloodmeal resulting in a massive uptake of haemoglobin and other proteins of haemolised blood by epithelial cells lining the gut wall. Consequently these epithelial cells are filled with electron-dense haemoglobin inclusions. In this study the inability to detect *Cowdria* organisms in gut epithelial cells or in the gut lumen after 3 days of feeding, could be ascribed to the presence of these haemoglobin inclusions masking single parasites that may at this stage have been released from readily detectable colonies. Jongejan (1990) demonstrated that parasitized cultured endothelial cells rupture and consequently release *C. ruminantium* organisms (Senegal and Welgevonden stocks) 5–6 days post-inoculation. Furthermore the life span of the different stocks of *C. ruminantium* vary (Jongejan, 1990).

Cowdria colonies were randomly distributed throughout the cytoplasm of the gut epithelial cells which is contrary to the findings of Bezuidenhout (1984) and Kocan *et al.* (1987b) who found colonies mainly confined to areas in the vicinity of the basement membrane. In this study no extracellular organisms were found in the gut lumen which differs from the results of Cowdry (1925). The number of *Cowdria* colonies infecting gut epithelial cells ranged from 1 to *c* 17 per cell and coincided with the number of colonies reported in endothelial cells *in vitro* (Bezuidenhout *et al.*, 1985).

The matrix in which the organisms were suspended was morphologically similar to the matrix described in the vertebrate host (Pienaar, 1970; Prozesky, 1987) and cultured endothelial cells (Prozesky *et al.*, 1986). In the present study there was no correlation between the matrix density and size of the organisms within a colony. This is in contrast with results in the vertebrate host where Pienaar (1970) described a well developed colony matrix in colonies containing small organisms and a decreasing matrix density in colonies with larger organisms.

Electron-dense, homogeneous inclusions were seldom identified in colonies on days 1 and 2 of feeding. They were comparable with the electron-dense inclusions described by Kocan *et al.* (1987a) in *Cowdria* colonies in the midgut epithelial cells of feeding nymphal *A. hebraeum* ticks. These authors suggested a possible dependency of the *Cowdria* organisms on the inclusions due to the fact that the outer membrane of some organisms adhered to the inclusion's surface. In this study the infrequent occurrence of the inclusions and the limited number of organisms that adhered to them made the assumption of a possible dependency unlikely.

Haemocytes of terrestrial arthopods are migratory cells that display phagocytic activity (Dolp, 1970; Brinton & Burgdorfer, 1971; Gupta, 1979; Amosova, 1983; Tsvileneva, 1961a and Crossley, 1975, cited by Amosova, 1983). They may aid in the dissemination of tick borne pathogenic agents such as certain *Rickettsiae* from the gut to various other tissues (Brinton & Burgdorfer, 1971; Amosova, 1983; Kocan & Bezuidenhout, 1987).

In the present study, small groups of *Cowdria* organisms occurred within the cytoplasm of a haemocyte. The presence of *Cowdria* within these cells could easily have been overlooked in light microscopical studies. The physiological state and activity of ticks influence the number and different types of haemocytes within them (Dolp, 1970; Brinton & Burgdorfer, 1971). Du Plessis (1985) found that only 1–2 % of the haemocytes in partially engorged adult *A. hebraeum* ticks were parasitized by *Cowdria*. There can be little doubt about the identity of the parasitized haemocyte in the present study, since there is no resemblance between the spherules of a haemocyte and any of the organelles of a mammalian leucocyte (Dolp, 1970; Brinton & Burgdorfer, 1971; Bloom & Fawcett, 1986; Gupta, 1979). The mottled appearance of the spherules displayed by the haemocyte in question, has been described as a feature of type III spherule haemocytes (Brinton & Burgdorfer, 1971). Furthermore, mammalian polymorphonuclear leucocytes are known to have lobulated nuclei, whereas haemocytes do not (Dolp, 1970; Brinton & Burgdorfer, 1971; Bloom & Fawcett, 1986). It is also known that haemocytes infiltrate the gut wall of ticks (Brinton & Burgdorfer, 1971). It is therefore suggested that *Cowdria* organisms can spread by means of haemocytes from the intestinal tract to other organs of the tick, including the salivary glands.

Reticulate bodies represented the principle morphological form of *C. ruminantium* within the gut epithelial cells. Single intermediate bodies which represent a developmental form between reticulate and elementary bodies in tissue culture (Jongejan, 1990), were identified on days 1 and 2 of feeding. No elementary bodies were observed. Bezuidenhout (1984) and Kocan *et al.* (1987b) observed only reticulate forms of the organism in the gut epithelial cells of adult *A. hebraeum* and adult *A. variegatum* ticks, respectively. Although Kocan *et al.* (1987a) described reticulate bodies as the dominant form of *Cowdria* in the gut and salivary gland acini epithelial cells in feeding *A. hebraeum* nymphae, elementary bodies were also present. Apparently the reticulate, intermediate and elementary bodies represent part of the developmental cycle of *Cowdria* in ticks (Kocan *et al.*, 1987a), cultured endothelial cells (Prozesky *et al.*, 1986; Jongejan, 1990) and in the vertebrate host (Prozesky, 1987).

Crystalline containing forms, occasionally observed in this study, were also described by Kocan *et al.* (1987a) in the gut epithelial cells of feeding *A. hebraeum* nymphae. Popov, Shatkin, Avakyan & Prozorousky (1977, cited by Avakyan & Popov, 1984) observed crystalline forms in *Rickettsiae* and *Chlamydiae* in tissue culture under unfavourable conditions. Crystalline inclusions have been observed in many different types of cells (Fawcett, 1966). It is known that virus-infected cells produce tubular structures in pseudo-crystalline forms which may be due to the assembly of one or more protein components produced in excessive amounts (Hine, 1988). A similar process may occur in some *Cowdria* organisms, the trigger mechanism of which is not clear.

Organisms were always surrounded by a well demarcated double membrane. Contrary to this, Prozesky *et al.* (1986) and Prozesky (1987) occasionally found *Cowdria* organisms in cultured endothelial cells that were enveloped by an electron-dense layer surrounded by a well demarcated fine fibrillar layer. These layers morphologically corresponded to the 'capsule' or slime layer present in many bacteria and rickettsiae (Avakyan & Popov, 1984). Rikihisa & Logan (1986) also described an enveloping capsule (peptidoglycan-like material) surrounding *C. ruminantium* organisms in the endothelial cells of the choroid plexus of goats that were infected IV with the Mali and Kümm stocks of *Cowdria*.

The majority of *Cowdria* colonies in the gut epithelial cells were located within a membrane-bound vacuole. On day 2 of feeding, however, multiple small groups of organisms, some of which were not membrane-bound, were seen in close proximity to a large colony or were scattered in the cell cytoplasm, indicating intracellular spread of organisms. These findings corroborate the observations of Prozesky (unpublished data, 1986, cited by Prozesky, 1987) in cultured endothelial cells.

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