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ULTRASTRUCTURAL FEATURES OF *COWDRIA RUMINANTIUM* IN MIDGUT EPITHELIAL CELLS AND SALIVARY GLANDS OF NYMPHAL *AMBLYOMMA HEBRAEUM*

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ABSTRACT

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Colonies of *Cowdria ruminantium* were studied in midgut epithelial cells and salivary gland acini of nymphal *Amblyomma hebraeum* that were infected experimentally as larvae. Colonies were found in both tissues and studied with light and electron microscopy. Colonies observed within gut cells frequently contained 2 types of the organism: electron-dense and reticulated forms. The morphology of colonies from salivary glands, as seen with light microscopy, varied from compact, densely-staining, small colonies to larger ones in which individual organisms were apparent. With electron microscopy, most organisms in salivary glands were reticulated and appeared to be dividing by binary fission. In both types of host cells, colonies often contained a dense inclusion to which reticulated organisms were adhered.

INTRODUCTION

Colonies of *Cowdria ruminantium* were first described in ticks by Cowdry (1925). They were demonstrated in gut epithelial cells and occasionally within the gut lumen. These findings were recently confirmed by fluorescent antibody staining, light and electron microscopy (Bezuidenhout, 1984). Colonies of *C. ruminantium* were also found in midgut epithelial cells of *A. variegatum* (Kocan, Morzaria, Voigt, Kiarie & Irvin, 1986).

The development of *C. ruminantium* in ticks has not been well understood. Different stages of the organism were identified in the vertebrate host and characterized as small, intermediate, large and very large forms (Pienaar, 1970). More recently, 3 forms of *C. ruminantium* were described in cell culture, including elementary and reticulated forms along with an intermediate stage of the organism (Prozesky, Bezuidenhout & Paterson, 1986). Thus far, the only stage of *C. ruminantium* observed in ticks has been a reticulated form.

Furthermore, the mechanism of transmission of *C. ruminantium* to the vertebrate host has not hitherto been described. Transmission by gut regurgitation has been suggested because organisms were not detected in salivary glands, and homogenates of these tissues from infected adult ticks that were unfed or fed for 2–3 days did not cause heartwater after intravenous injection into susceptible goats (A. J. Winkelhoff, unpublished report, 1979, cited by Uilenberg, 1983).

In more recent studies, saliva collected from infected ticks was sometimes infective, and salivary gland homogenates were consistently infective for susceptible sheep (Bezuidenhout, 1981; J. D. Bezuidenhout, unpublished data, 1986), thus suggesting that these tissues are involved in parasite development.

The present study was undertaken to study *C. ruminantium* in the gut cells and salivary glands of *A. hebraeum* nymphae that had been exposed to heartwater as larvae using light and electron microscopies. The 2 forms of the organism found in colonies in gut cells are described, suggesting a developmental cycle of this organism in the invertebrate host. The morphology of *C. ruminantium* in the salivary glands of nymphae that had fed is described in this report, and the importance of the finding is discussed.

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MATERIALS AND METHODS

Ticks

Amblyomma hebraeum nymphae were infected as larvae with the Ball 3 strain of *Cowdria ruminantium*, as described previously (Bezuidenhout, 1981). Uninfected nymphae from the same strain were studied as controls. Infected and control nymphae were allowed to feed on susceptible sheep, which were monitored daily for clinical heartwater.

Collection of tissues and electron microscopy

Tick tissues were collected from unfed infected and control nymphae, as well as from ticks that were allowed to feed on Days 1–5. On each collection day, the internal organs of nymphae were fixed by pushing the tissue from excised bodies of ticks into cold, 2 % glutaraldehyde in a 0,2 M sodium cacodylate buffer with 0,5 % sucrose. The tissues were post-fixed in 2 % osmium tetroxide in the same buffer and processed for electron microscopy, according to the procedures of Kocan, Venable & Brock (1978). Thick sections (1 μm) were stained with Mallory's stain (Richardson, Jarrett & Finke, 1960) for 2 min at 60 °C. Ultrathin (silver reflective) sections were cut with an ultramicrotome and diamond knife. The sections were collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1978) and observed and photographed with an electron microscope.

RESULTS

Tick transmission studies

Nymphae fed as larvae on heartwater-infected sheep transmitted *Cowdria ruminantium* to a susceptible sheep. The sheep had a temperature reaction from 9 days after infestation with ticks. After tetracycline therapy and recovery from clinical symptoms, the sheep was challenged with 5 mℓ of infective blood vaccine and found to be immune to heartwater. The sheep, fed on by control nymphae, did not develop heartwater disease and was found to be susceptible upon challenge.

Light microscopy studies of midgut epithelial cells

Colonies of *C. ruminantium* were present in sections of midgut epithelial cells of unfed infected nymphae, as well as in those collected on Days 1–4 of feeding. Colonies were not observed in ticks collected on Day 5 of feeding. Colonies were not seen in tissues of control ticks.

Electron microscopy studies of midgut epithelial cells

Colonies of *C. ruminantium* in midgut epithelial cells of nymphae contained both electron-dense and reticulated forms of the organism (Fig. 1a & b). The organisms were always within a parasitophorus vacuole. An

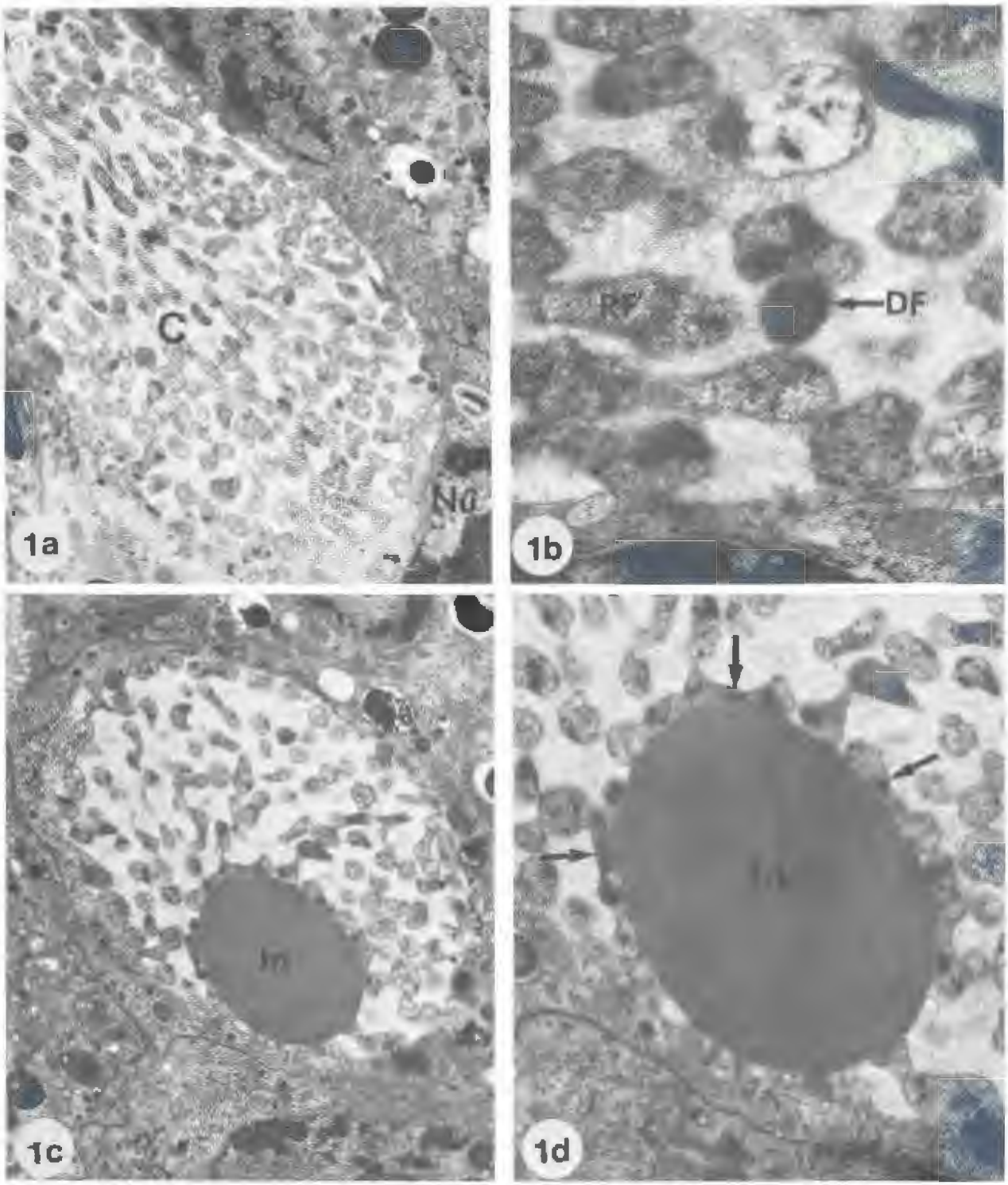

FIG. 1 Electron micrographs of *C. ruminantium* in midgut epithelial cells of nymphs that fed as larvae on an infected sheep

a Colony (C) within a midgut epithelial cell adjacent to 2 host nuclei (Nu): × 5 000

b A high magnification of reticulated (RF) and electron-dense forms (DF) of *C. ruminantium* within a colony: × 20 000

c A colony containing an electron-dense inclusion (In) with *C. ruminantium* organisms adhering to the surface: × 6 000

d Higher magnification of a colony inclusion (In) with *C. ruminantium* organisms adhering to the surface (arrows): × 8 000

electron-dense inclusion was often seen within colonies, and organisms adhered to the surface of the inclusions (Fig. 1c & d). Electron-dense forms of the organisms often had a crystalline formation in which the pattern of the formation varied (Fig. 2a & b).

Light microscopy studies of salivary glands

Colonies of *C. ruminantium* were observed in salivary gland acini in 4 out of the 20 nymphae that had fed for 4 days (Fig. 3a–d). The diameter of the colonies ranged from 5 μm to 30 μm, and the morphology varied from smaller, densely-staining colonies to larger colonies in which individual organisms were clearly visible (Fig. 3a–d). The colonies were seen also in simple granular cells of types II and III acini. Colonies were not seen in tissues of corresponding control ticks.

Electron microscopy studies of salivary glands

Most colonies of *C. ruminantium* in salivary glands of *A. hebraeum* contained reticulated forms of the orga-

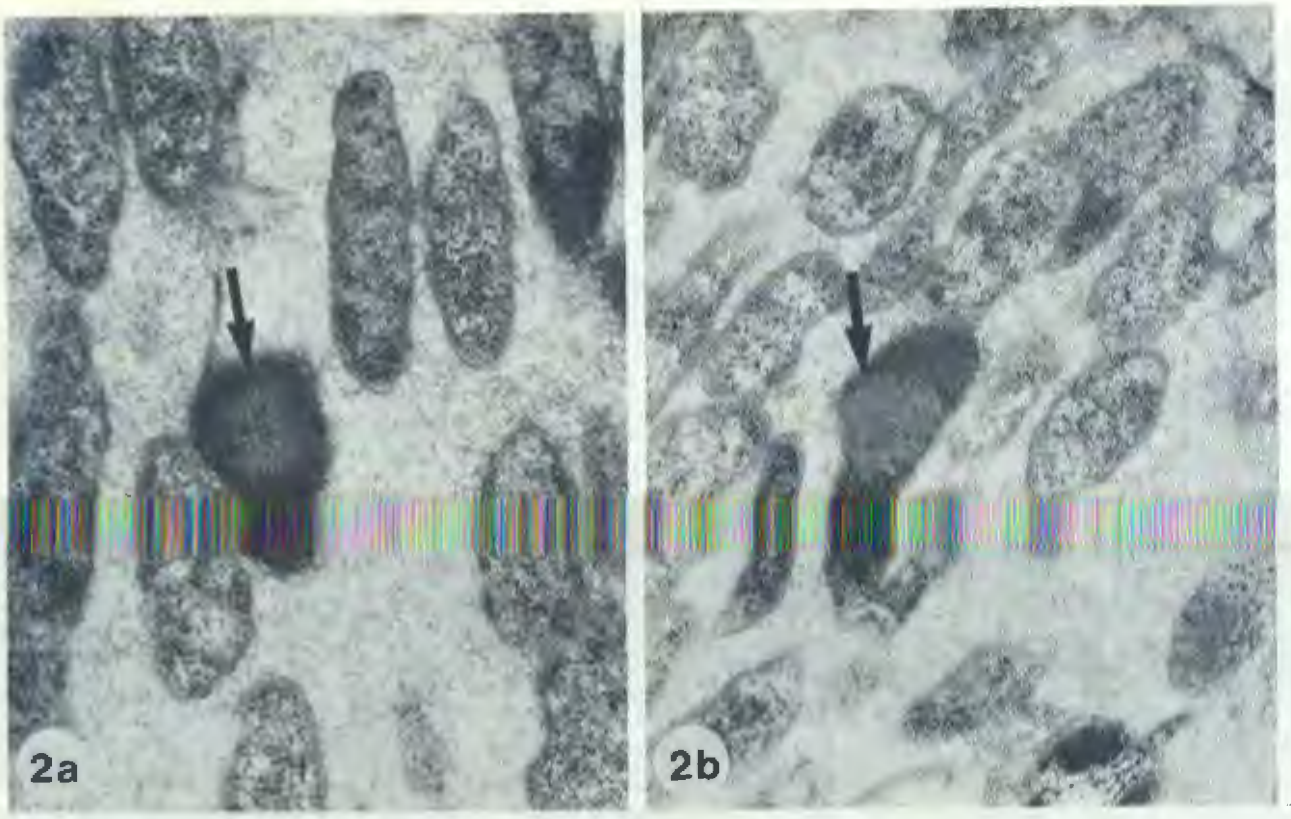

FIG. 2 a&b Electron micrographs of electron-dense forms of *C. ruminantium* that contain a crystalline formation (arrows): × 20 000

nisms that appeared to be dividing by binary fission (Fig. 4a–d). A few colonies appeared to contain denser forms of the parasite (Fig. 4b). Often colonies contained organisms with electron-dense cores (Fig. 4c). Smaller colonies were densely packed with reticulated organisms (Fig. 4d). Often a moderately dense material was seen within colonies in salivary glands (Fig. 4c and 5a and b). The outer membrane of some *C. ruminantium* organisms adhered to the surface of this material (Fig. 4c & 5a and b).

Discussion

In previous studies on *Cowdria ruminantium* in colonies within midgut epithelial cells of adult *Amblyomma* ticks, the organisms observed were reticulated forms that appeared to be dividing by binary fission (Bezuidenhout, 1984; Kocan *et al.*, 1986). Reticulated forms were also most often observed within colonies in salivary glands of nymphae, although some electron-dense forms were also present. In contrast, colonies of *C. ruminantium* in nymphal midgut observed in the present study contained both electron-dense and reticulated forms of the organism. The 2 forms may have been evident in nymphae because they were feeding, thus the colonies and organisms within may have been developing more rapidly.

The forms of *C. ruminantium* within colonies in *A. hebraeum* nymphae are similar to those described in endothelial cells of the vertebrate host by Pienaar (1970), and, more recently, in cell culture studies (Prozesky *et al.*, 1986). Both electron-dense and reticulated forms of the organism appear to be part of the developmental sequence of *Cowdria* in the vertebrate host, in ticks and *in vitro*. It has not been determined whether the electron-dense form is the stage that can survive extracellularly and can infect cells, as has been clearly demonstrated with the chlamydial organisms (Storz & Spears, 1977). The reticulated forms appear to be the predominant vegetative stages within cells because of the morphologic evidence of binary fission in mammalian and invertebrate cells (Bezuidenhout, 1984; Kocan *et al.*, 1986).

In these studies, dense inclusions were observed within colonies and were similar to those described previously by Kocan *et al.* (1986) in gut colonies of *A. variegatum*. These inclusions were also similar in morphology and staining to haemoglobin deposits normally seen within gut cells of ticks. The adherence of organisms to these inclusions suggests a dependence of the organism on this material. The organisms within colonies in salivary glands of the same group of nymphae also adhered to an inclusion, but it was of moderate stain intensity and did not appear to be the same as the inclusions observed in the gut.

Crystalline formations were seen only in electron-dense forms of *Cowdria* in gut colonies. It is not known whether the inclusions may play a role in the developmental cycle, making it possible for this stage to resist environmental changes, or may represent an abnormal form.

This report is the first description of *C. ruminantium* in salivary glands of known infective, transmitting ticks. This finding confirms observations of previous studies in our laboratory in which salivary gland homogenates and oral secretions proved to be infective for susceptible sheep (Bezuidenhout, 1981; J. D. Bezuidenhout, unpublished data, 1986). It is difficult to explain why these colonies have escaped recognition by previous investigators. The morphology of colonies in both light and electron microscopy is similar to that of colonies described in midgut epithelial cells (Cowdry, 1925; Bezuidenhout, 1984; Kocan *et al.*, 1986). However, the 1 μm plastic sections from the present studies provided better resolution of cell morphology, thus enhancing the differentiation of colony morphology from the many inclusions and granules that normally occur in salivary glands.

Colonies were seen only in salivary glands of nymphae that had fed for 4 days. The colonies were not observed to be ruptured, nor were organisms observed outside of the inclusion membrane or within associated collecting ducts. Nonetheless, we believe that development of *C. ruminantium* in salivary glands is followed

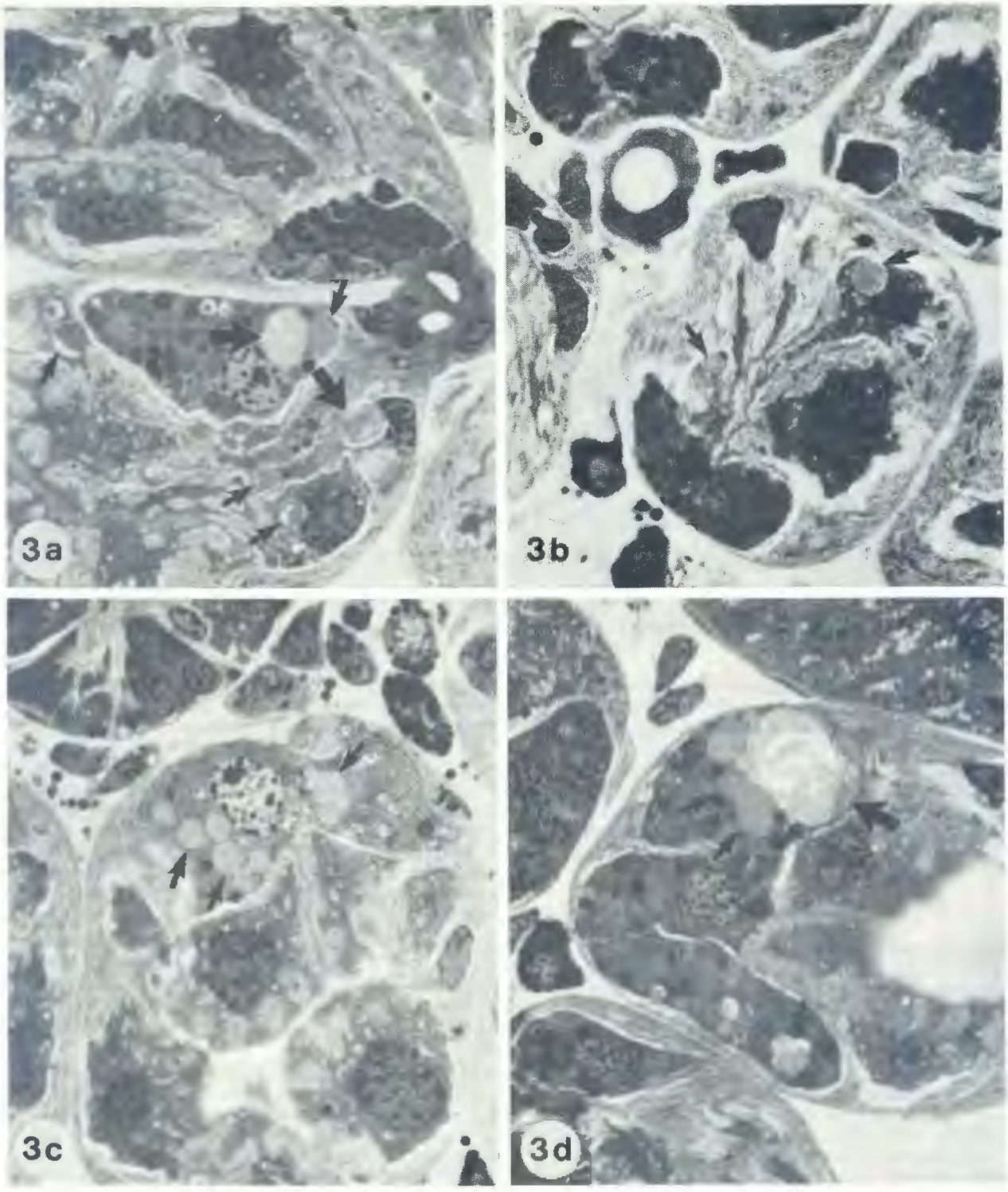

FIG. 3 a–d Photomicrographs of *C. ruminantium* in cross-sections of salivary gland acini from nymphal *A. hebraeum* which had fed for 4 days. The 1 μm plastic sections are stained with Mallory's stain: × 2 500. Colonies vary in morphology from small, densely-staining colonies (small arrows) to larger colonies with separated organisms (large arrows)

by transmission of the organism via this organ to the vertebrate host. Furthermore, this mechanism of transfer appears to be co-ordinated with the feeding cycle of the tick.

The development of rickettsial organisms in ticks has not been as well documented as for the protozoans. Perhaps this resulted because *Rickettsia*, the type genus for the order Rickettsiales, has been reported to divide only by binary fission and to infect all tick tissues. The infection is referred to as "generalized", and does not involve a specific sequence of development (Burgdorfer & Varma, 1968). However, different stages of *Cowdria* have been observed in tick tissues and in cell cultures (Kocan *et al.*, 1986; Prozesky *et al.*, 1986), and similar findings have been reported in the complex development of *Anaplasma marginale* in a *Dermacentor* tick sp. (Kocan, 1986). It appears that these parasites initially develop within the tick gut cells, followed by subsequent stages invading and developing in salivary gland cells. The development of the transmitted stage occurs as the ticks feed—a process that has been well-documented for the protozoans, *Babesia* (Riek, 1968; Mahoney, 1977) and *Theileria* (Mehlhorn & Schein, 1984).

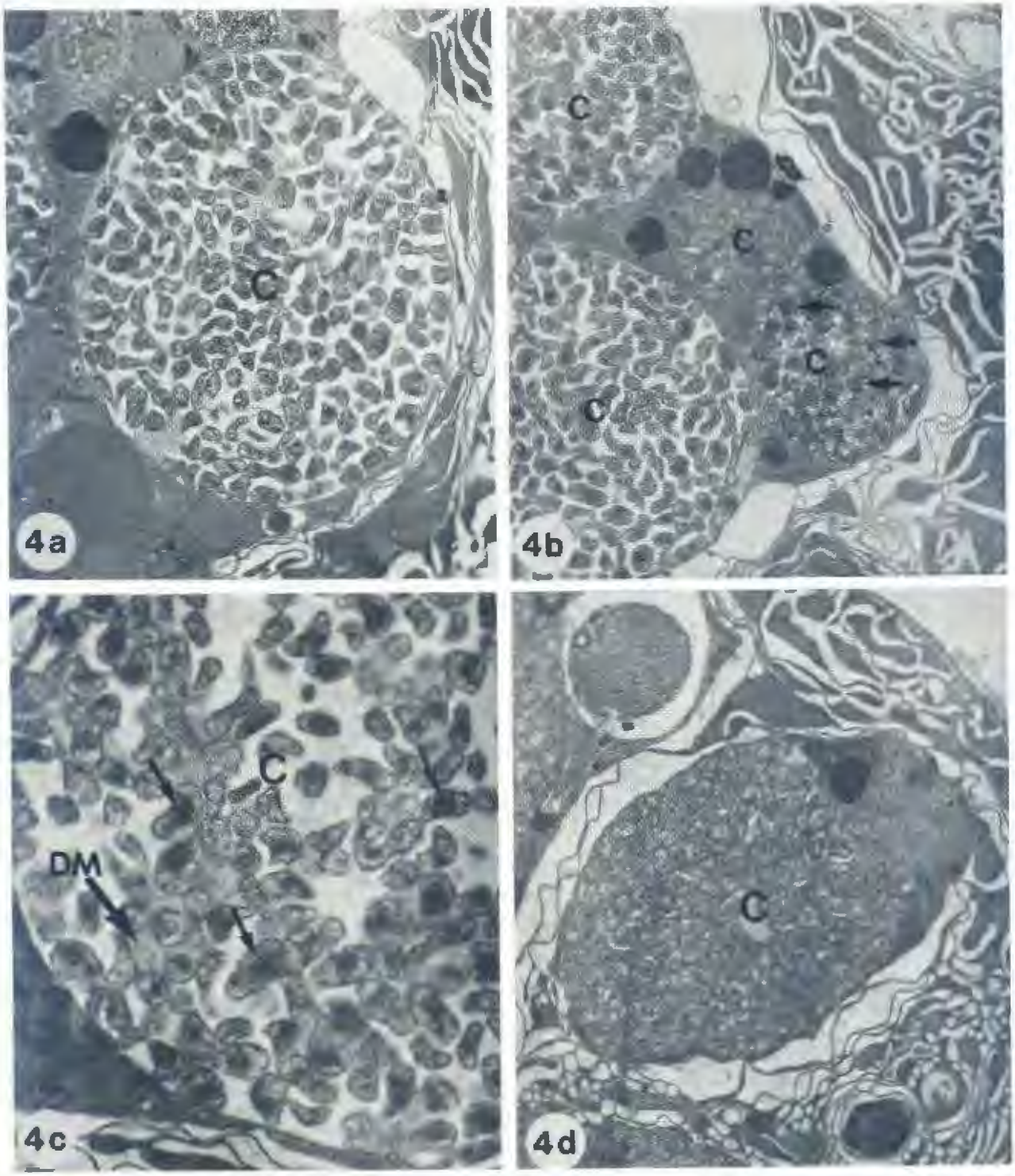

FIG. 4 a–d Electron micrographs of *C. ruminantium* in salivary gland acini of *A. hebraeum* that fed for 4 days

a A large colony (C) within a simple granulated cell of a salivary gland acinus containing reticulated forms of the organisms: × 6 000

b 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

c A colony (C) of *C. ruminantium* that contains reticulated organisms with electron-dense centres (arrows). Dense material (DM) is between some of the organisms: × 10 000

d A colony (C) of *C. ruminantium* that contains densely-packed, reticulated organisms: × 6 000

Further evidence of this sequential development of *Cowdria* in ticks is the infectivity of other tick tissues, including homogenates of hypodermal tissues, haemolymph malpighian tubules and rectal ampoules of prefed adult *A. hebraeum* (J. D. Bezuidenhout, unpublished data, 1986). Du Plessis (1985) also demonstrated *Cowdria* in tick haemocytes.

Salivary gland stages were observed in tissues similar to those used for tick-derived heartwater vaccine. Fully-engorged nymphae that were infected as larvae were highly infective; only 0,0015 homogenized nymph was necessary to cause infection in susceptible sheep (Bezuidenhout, 1981). These data suggest that the salivary gland stages may be highly infective.

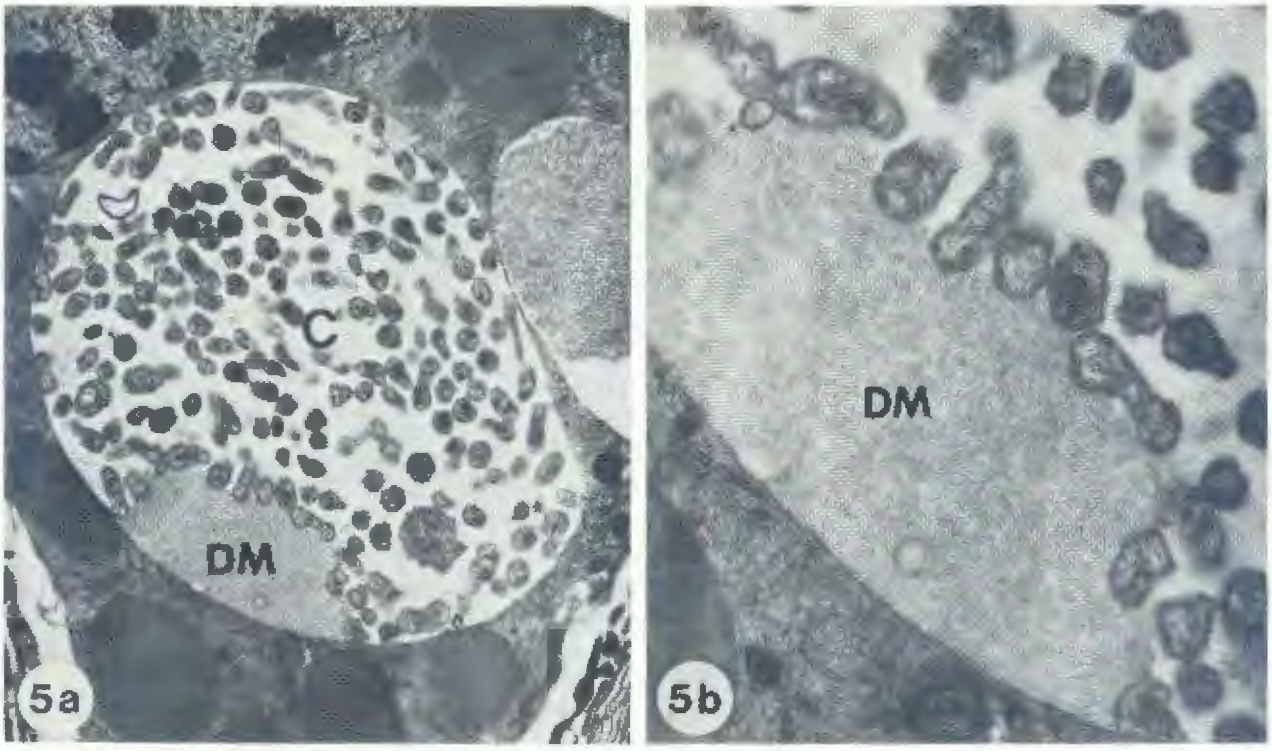

FIG. 5 a&b Electron micrographs of colonies of *C. ruminantium* in a salivary gland cell of nymphal *A. hebraeum* that had fed for 4 days

a A colony (C) containing a moderately dense material (DM): × 5 000

b A higher magnification of the dense material (DM) with *C. ruminantium* organisms attached to the surface: × 15 000

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REFERENCES

BEZUIDENHOUT, J. D., 1981. The development of a new heartwater vaccine, using *Amblyomma hebraeum* nymphae infected with *Cowdria ruminantium*. *In:* WHITEHEAD, G. B. & GIBSON, J. D. (eds.). Tick biology and control. pp. 41–45. Tick Research Unit, Rhodes University, Grahamstown.

BEZUIDENHOUT, J. D., 1984. Demonstration of *Cowdria ruminantium* in *Amblyomma hebraeum* by fluorescent antibody techniques, light and electron microscopy. *Onderstepoort Journal of Veterinary Research*, 51, 213–215.

BURGDORFER, W. & VARMA, M. G. R., 1968. Transstadial and transovarial development of disease agents in arthropods. *Annual Review of Entomology*, 12, 347–376.

COWDRY, E. V., 1925. Studies on the aetiology of heartwater. II. *Rickettsia ruminantium* (n. sp.) in the tissues of ticks transmitting the disease. *Journal of Experimental Medicine*, 42, 253–274.

DU PLESSIS, J. L., 1985. The natural resistance of cattle to artificial infection with *Cowdria ruminantium*. The role played by conglutinin. *Onderstepoort Journal of Veterinary Research*, 52, 273–277.

KOCAN, KATHERINE M., 1986. Development of *Anaplasma marginale* in ixodid ticks: co-ordinated development of a rickettsial organism and its tick host. *In:* SAUER, J. & HAIR, J. A. (eds.). Morphology, behavioral ecology and physiology of ticks. England: Ellis Horwood Inc. pp. 472–505.

KOCAN, KATHERINE M., VENABLE, J. H. & BROCK, W. E., 1978. Ultrastructure of anaplasmal inclusions (Pawhuska isolate) and their appendages in intact and hemolyzed erythrocytes and in complement fixation antigen. *American Journal of Veterinary Research*, 39, 1131–1135.

KOCAN, KATHERINE M., MORZARIA, S. P., VOIGT, W. P., KIARIE, J. & IRVIN, A. D., 1986. Demonstration of colonies of *Cowdria ruminantium* in midgut epithelial cells of *Amblyomma variegatum*. *American Journal of Veterinary Research* (in press).

MAHONEY, D. F., 1977. Babesia of domestic animals. *In:* KREIER, J. P. (ed.). Parasitic protozoa. 4, 1–52. New York & London: Academic Press.

MEHLHORN, H. & SCHEIN, E., 1984. The piroplasms: life cycle and sexual stages. *Advances in Parasitology*, 23, 37–103.

PIENAAR, J. G., 1970. Electron microscopy of *Cowdria (Rickettsia) ruminantium* (Cowdry, 1926). in the endothelial cells of the vertebrate host *Onderstepoort Journal of Veterinary Research*. 37, 67–78.

PROZESKY, L., BEZUIDENHOUT, J. D. & PATERSON, CAMILLA L., 1986. Heartwater. An *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort Journal of Veterinary Research* (in press).

RICHARDSON, K. C., JARRETT, L. & FINKE, E. H., 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technology*, 35, 313–323.

RIEK, R. F., 1968. Babesiosis. *In:* WEINMAN, D. & RISTIC, M. (eds.). Infectious blood diseases of man and animals. 219–268. New York: Academic Press.

STORZ, J. & SPEARS, P., 1977. Chlamydiales: Properties, cycle of development and effect on eukaryotic host cells. *Current Topics in Microbiology and Immunology*, 76, 168–214.

UILENBERG, G., 1983. Heartwater (*Cowdria ruminantium* infection): Current status. *Advances in Veterinary Science of Comparative Medicine*, 27, 427–480.

VENABLE, J. H. & COGGESHALL, R., 1978. A simplified lead citrate stain for use in electron microscopy. *Journal of Cellular Biology*, 25, 407–408.

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