AN ULTRASTRUCTURAL STUDY OF THE DEVELOPMENT OF *BABESIA OCCULTANS* IN THE SALIVARY GLANDS OF ADULT *HYALOMMA MARGINATUM RUFIPES*

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ABSTRACT

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The development of *Babesia occultans* in the salivary glands of adult *Hyalomma marginatum rufipes* was studied with the electron microscope. Sporogony involved a process of multiple fission in which sporozoites formed from the periphery of a polymorphous sporont. Different stages of development were found concurrently in individual acini as well as within individual acinar cells. Mature sporozoites were found on Day 3 post-tick attachment and measures 3,0–3,5 × 1,5 μm. The apical complex consisted of a polar ring and 4–6 rhoptries. Micronemes were concentrated anteriorly and 1 or 2 spherical bodies were identified in each sporozoite. The general pattern of development was similar to that described in several other *Babesia* spp. but distinct morphologic differences were noted.

INTRODUCTION

Until recently, *Babesia bigemina* and *Babesia bovis* were the 2 *Babesia* spp. known to infect cattle in South Africa. Both are transmitted to cattle by ticks of the genus *Boophilus* (Potgieter & Els, 1976; 1977) and they continue to be of economic importance to the cattle industry (De Vos, 1979). In 1981, Thomas & Mason isolated a previously undescribed *Babesia* sp. from *Hyalomma marginatum rufipes* ticks collected from cattle in the Northern Transvaal. They subsequently transmitted it to susceptible cattle. The piroplasms were morphologically similar to both *B. bovis* and *B. bigemina* but produced only mild clinical reactions even in splenectomized animals. Gray & De Vos (1981) determined that this species was serologically distinct from other *Babesia* spp. in South Africa and had a wide distribution within the country. They subsequently named the organism *Babesia occultans*. Recent investigations at this institute into the serological relationship of *B. occultans* to *B. bigemina* and *B. bovis* indicate that there may be significant cross reactivity between certain strains of the 2 pathogenic species and *B. occultans* (M. P. Combrink, unpublished data, 1987). This has led to some confusion regarding the status of this new species. Investigations are currently under way to elucidate the antigenic relationship between *B. occultans* and these other species.

In this study the ultrastructural features and development of *B. occultans* in its tick vector was investigated. The development of *B. bigemina* and *B. bovis* in their tick vectors has been previously described (Potgieter & Els, 1976; 1977). The development of *B. equi* in the related tick species *Hyalomma anatolicum anatolicum* and *Hyalomma anatolicum excavatum* has also been described by Moltmann, Mehlhorn, Schein, Voigt & Friedhoff (1983). This study was conducted to determine the pattern of development at the ultrastructural level of *B. occultans* and relate it to that described in other *Babesia* spp.

METHODS AND MATERIALS

Infection of ticks

A splenectomized bovine was inoculated with 5 mℓ of a *B. occultans* blood stabilate collected from a tick-infected bovine carrier. Beginning on the day of inoculation, the animal was injected with 0,05 mg/kg of corticosteroids[1] for 5 days to enhance the development of a parasitaemia. Uninfected, adult *H. m. rufipes*, isolated from a bovine in the Potgietersrus area and maintained at

this institute since 1985 were fed on both ears of the parasitaemic bovine. The highest parasitaemia during tick feeding was 0,34 organisms per 100 red blood cells. Engorged female ticks were collected and allowed to lay eggs. Infected ticks were identified through the detection of parasites in Giemsa-stained haemolymph smears. The larvae and nymphae were fed on rabbits and engorged nymphae were allowed to moult. The subsequent adults were fed on a clean, susceptible bovine and, beginning with unfed adults, were collected on each day of feeding. Tissues were collected and fixed for electron microscopy, as described below. Clean, adult *H. m. rufipes* were fed on a non-infected bovine and ticks were collected on each feeding day as controls.

Electron microscopic procedures

Immediately after collection, the ticks were dissected in RPMI 1640 medium[2] and the salivary glands were removed and placed into cold 2 % glutaraldehyde in 0,2 M sodium cacodylate buffer with 0,5 % sucrose (pH 7,4). The glands were post-fixed in 2 % osmium tetroxide in 0,2 M sodium cacodylate buffer, dehydrated in a graded ethanol series, passed through propylene oxide as a transitional solvent and embedded in Dow Epoxy Resin (DER[3]). Thick sections of lum were cut and stained with Mallory's stain, according to Richardson, Jarret & Finke (1960) and examined for parasitic stages with a light microscope. Ultrathin sections (silver reflective) were cut on an ultramicrotome with a diamond knife and placed onto 300 mesh copper grids. The sections were stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1978) and viewed with a JOEL JEM1200EX electron microscope.

RESULTS

The first kinetes were observed in the salivary glands on Day 3 post attachment. They were located in granular acinar cells and were bounded by a pellicle composed of an outer plasmalemma and a dense inner layer (Fig. 1 & 2). The first stages to be identified were spherical forms enclosed within a double-unit, membraned, parasitophorous vacuole. Folded forms which appeared to be elongated and tapering were also seen (Fig. 1). Kinetes then became more spherical in shape and were found in direct contact with the host cell cytoplasm (Fig. 2). No transitional stages demonstrating the fate of the vacuolar membranes could be identified. The spherical kinetes
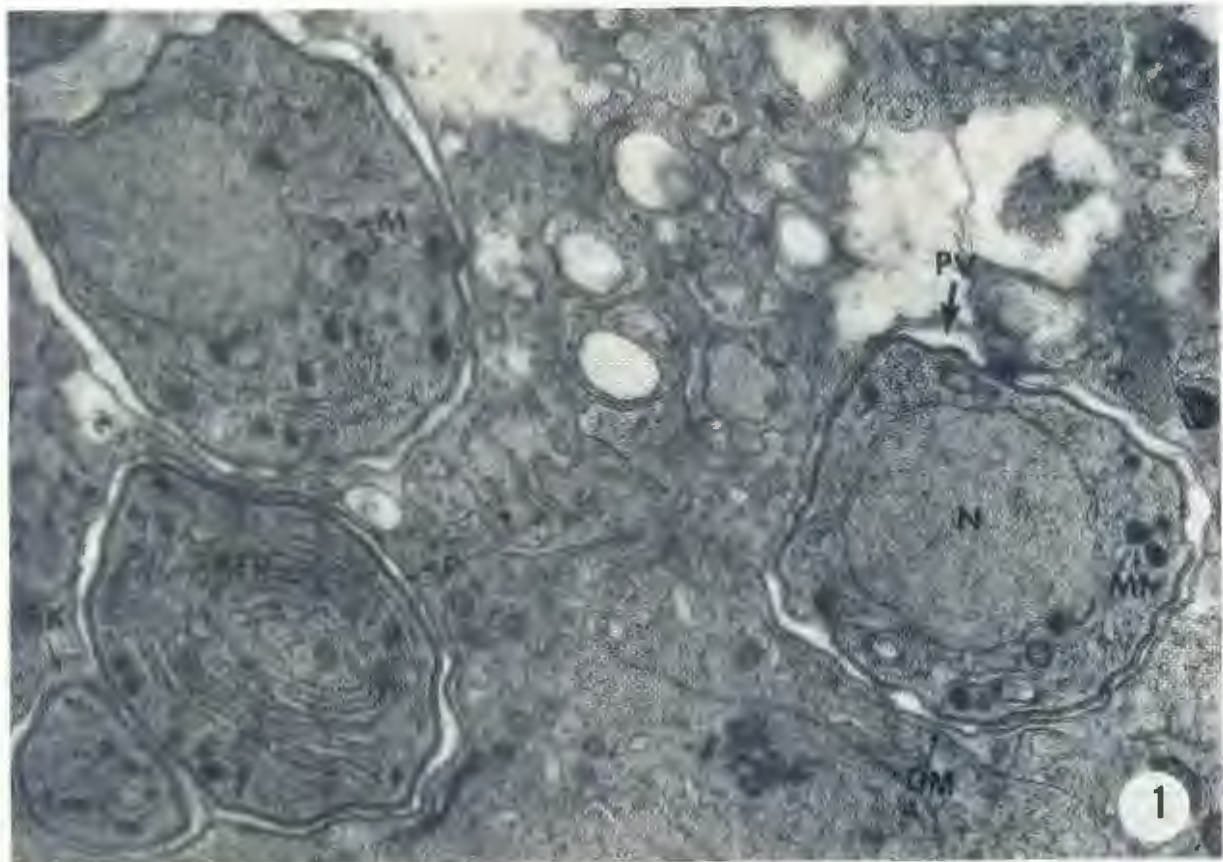

FIG. 1–2 Electron micrographs of developmental stages of *Babesia occultans* in the salivary glands of *Hyalomma marginatum rufipes*

    1   Cross-section through 3 kinetes. The organisms are enclosed in a parasitophorous vacuole (PV) composed of a double-unit membrane (DM) and contain a nucleus (N), mitochondria (M), micronemes (Mn), abundant rough endoplasmic reticulum (RER) and are surrounded by a double-membraned pellicle (P). Note the folded kinete (FK): × 12 000

    2   Longitudinal section through a kinete after the disappearance of the parasitophorous vacuole. The double-membraned pellicle, composed of an outer plasmalemma (PL) and a dense innerlayer (DL), is in direct contact with the host cell cytoplasm. The apical complex (AC) is still intact and subpellicular microtubules (Mt) can be seen radiating from this region. A multilamellar, coiled organelle (MCD) is also present: × 15 000

measured 2,5–3,0 μm in length and averaged 1,6 μm in width and had an anterior polar ring to which microtubules were attached in a rib-like arrangement (Fig. 2). Micronemes were scattered throughout the parasite cytoplasm along with mitochondria, well-developed, endoplasmic reticulum, ribosomes and a double-membraned nucleus (Fig. 1 & 2). Coiled, multilamellar structures were occasionally observed (Fig. 2). Multiple infected cells were commonly encountered and up to 10 kinetes were found in an individual acinar cell. Different cells of the same acinus were infected concurrently and numerous infected acini were found in infected ticks. Kinetes
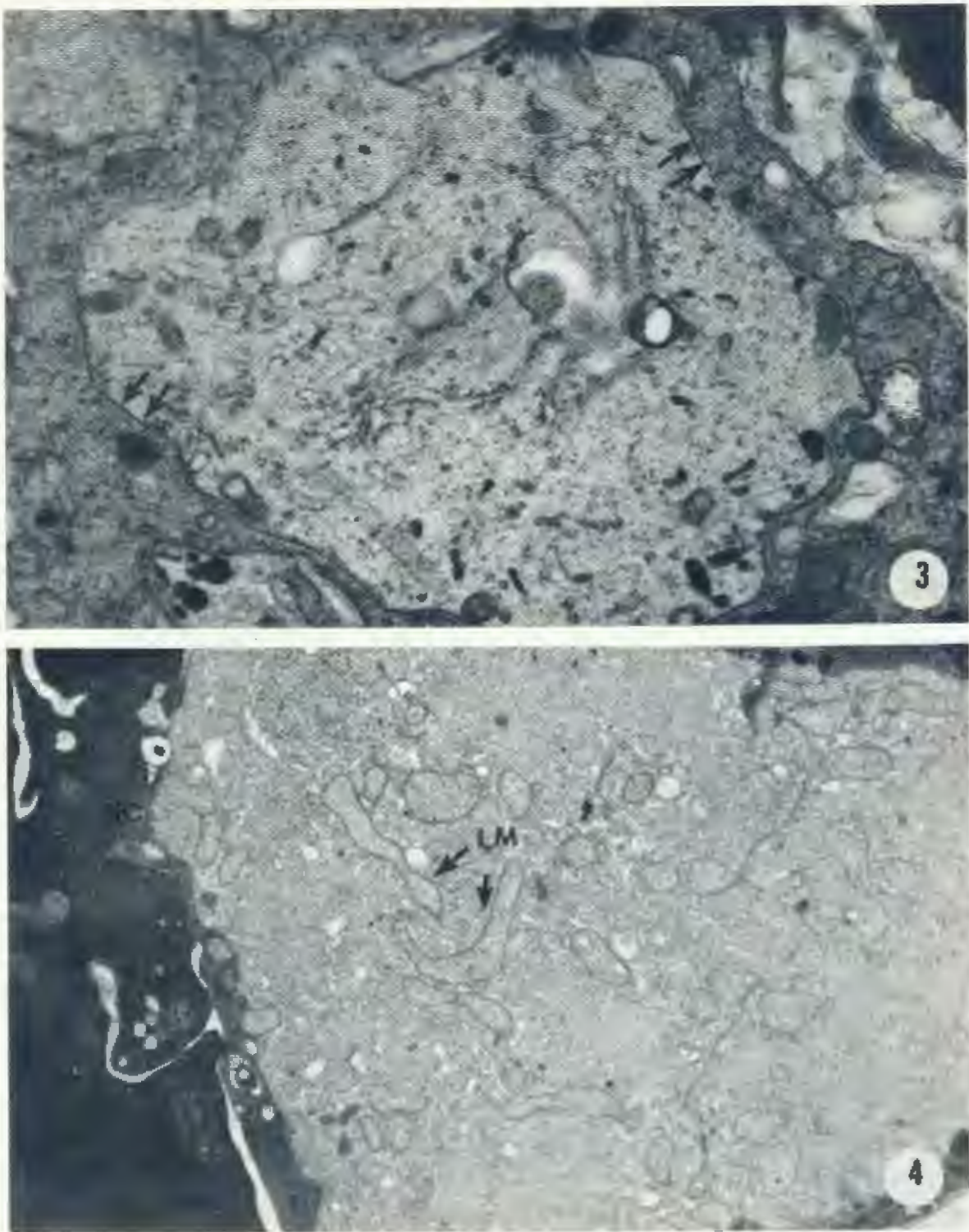
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FIG. 3–4 Electron micrographs of developmental stages of *Babesia occultans* in the salivary glands of *Hyalomma marginatum rufipes*

   3   Cross-section through a de-differentiating kinete undergoing sporont formation. Much of the pellicle has been reduced to a single membrane (double arrows) as the kinete losses its spherical shape. × 15 000

   4   Cross-section through a polymorphous sporont expanding through the host cell (HC). Note the limiting membrane (LM) of the parasite which has folded over itself many times. × 3 000

were found in the salivary glands on each successive day of tick feeding, including at engorgement on Day 8.

Kinetes underwent a process of de-differentiation, beginning with the loss of the osmiophylic layer of the pellicle and the apical complex. Cytoplasmic protrusions transformed the kinete into a polymorphous sporont (Fig. 3). The sporont which was bounded by the plasmalemma only expanded through the host cell cytoplasm, folding over itself while increasing in size (Fig. 4). The nucleus became highly lobulated and mitochondria were still prominent within the parasite cytoplasm (Fig. 5). The expanding sporont did not appear to induce host cell hypertrophy and remained within the host cell cytoplasm.

Karyokinesis occurred during the next phase of development but the point at which this occurred could not be
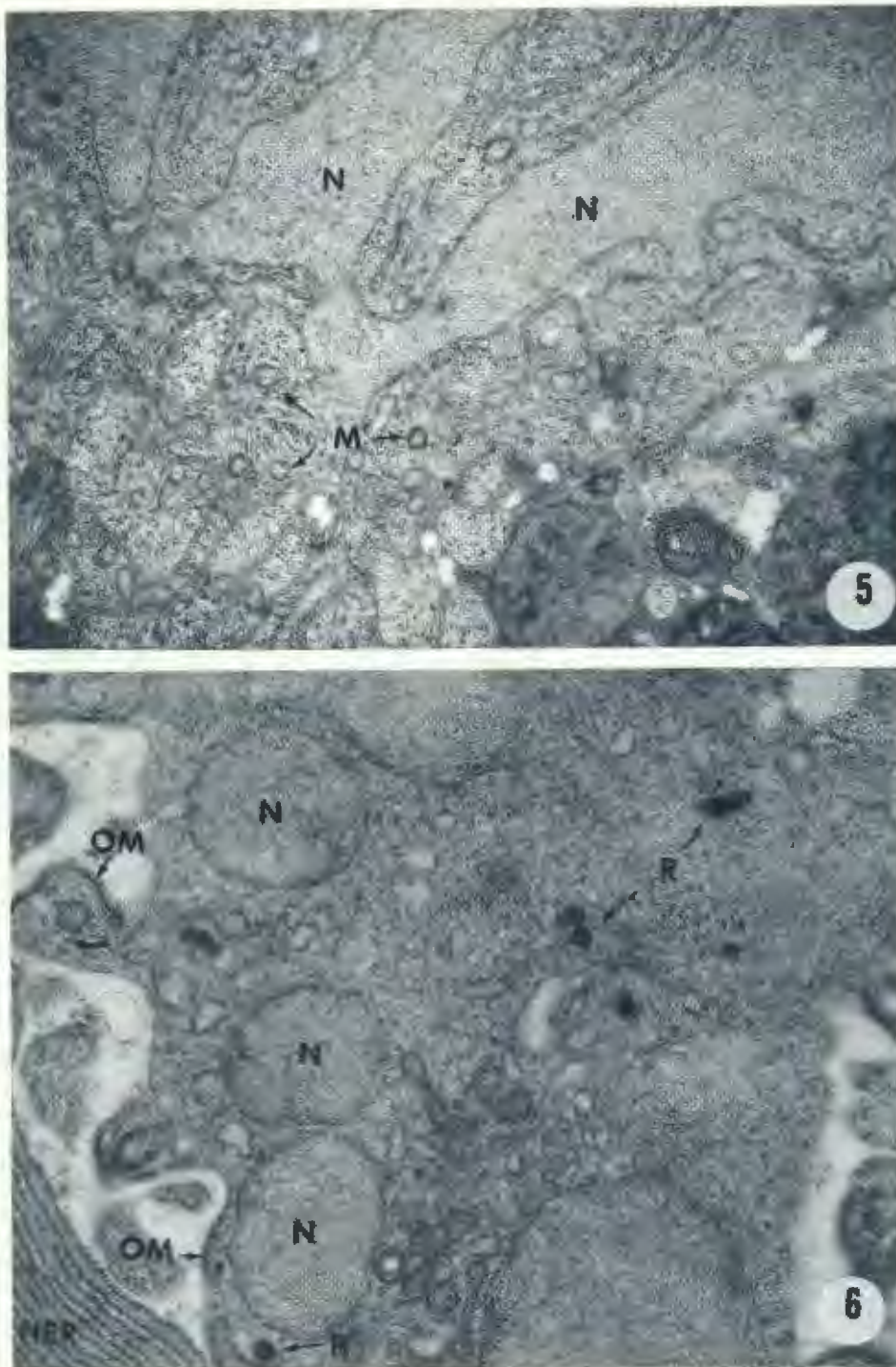
FIG. 5–6 Electron micrographs of developmental stages of *Babesia occultans* in the salivary glands of *Hyalomma marginatum rufipes*

5 Cross-section through a developing sporont in which the parasite nucleus (N) has become highly lobulated. Numerous parasite mitochondria (M) are also present. × 12 000

6 Cross-section through a sporont following karyokinesis. Note the peripherally located nuclei (N), sections of dense osmiophilic membrane (OM) and rhoptry precursors (R) that have formed. The sporont is surrounded by host cell endoplasmic reticulum (HER). × 15 000

determined. Many spherical nuclei of varying sizes were then observed, especially along the periphery of the sporont mass (Fig. 6). At a point just above each peripheral nucleus a dense osmiophilic layer formed just under the plasmalemma and a cytoplasmic projection then formed (Fig. 6). Densely staining structures, thought to be rhoptry precursors, were present near the protrusion as well as within the sporont mass (Fig. 6). Nuclei were incorporated into the cytoplasmic protrusions along with rhoptry precursors and the osmiophilic layer continued to form

through the developing sporozoite. This was then attached to the sporont mass by a narrower, neck-like region (Fig. 7). The immature sporozoites eventually pinched off from the mass. As the process continued the sporont mass became divided into smaller portions from which immature sporozoites continued to form. Residual parasite material, containing some large nuclei up to 2 μm in diameter, remained along with numerous mitochondria (Fig. 8a). Multilamellar structures were also found in the residual sporont material (Fig. 7). Follow-
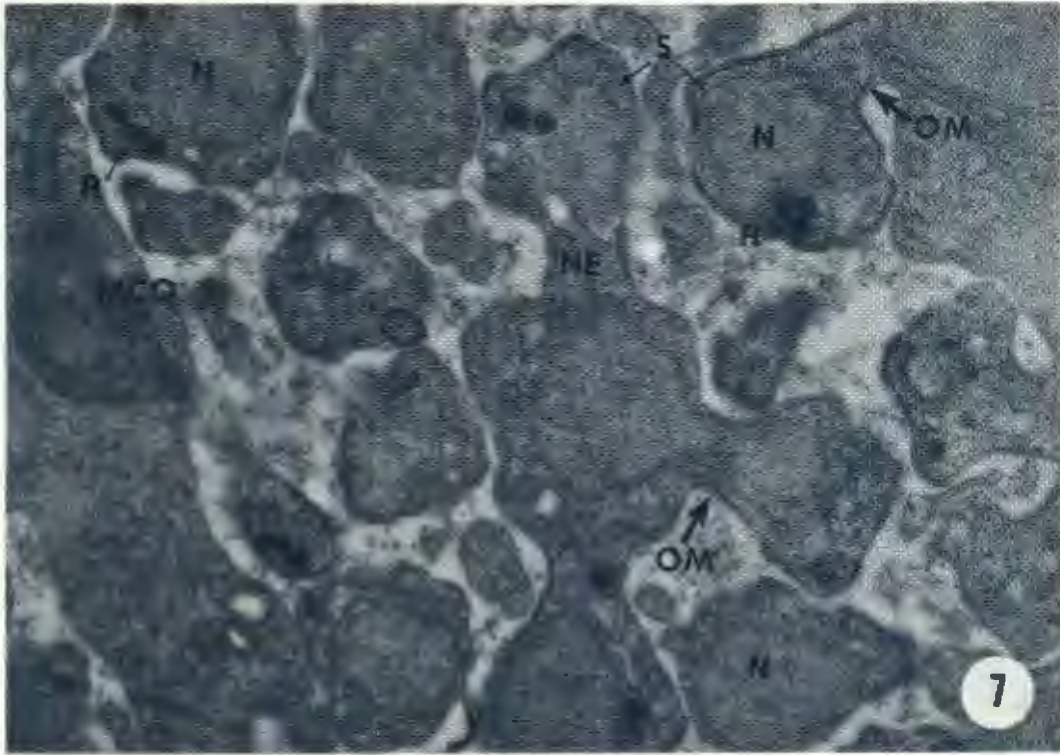
FIG. 7 Electron micrograph of a cross-section through a differentiating sporont of *B. occultans* in a salivary gland cell of *Hyalomma marginatum rufipes* from which individual sporozoites are forming. The sporozoites (S) form as cytoplasmic protrusions incorporating a nucleus (N) and rhoptry precursors (R). As the osmiophilic membrane (OM) extends into a neck-like region (NE), immature sporozoites "pinch off" from the parasite mass. Multilammelar, coiled organelles (MCO) are also present. × 11 000

ing separation, immature sporozoites increased in size and became rounded and the anterior polar ring formed from the 2 membranes of the osmiophilic layer (Fig. 8a & b). In some, rhoptries were present in the apical complex and micronemes were observed for the 1st time (Fig. 8a). Small granular spherical bodies were also observed (Fig. 8a).

Mature sporozoites were also first detected on Day 3 post-attachment and on each successive day of feeding. Mature sporozoites were pyriform, being broader at the apical end, and measured 3,0–3,5 μm in length with an average width of 1,5 μm. Parasite nuclei averaged 1 μm in diameter. The spherical bodies were bounded by a single membrane and there were often 2 per sporozoite with 1 being larger than the other (Fig. 9). The larger spherical bodies were 0,7 μm in diameter and were more electron-dense than those observed in immature sporozoites. Mature sporozoites also contained mitochondria, micronemes, which tended to be concentrated anteriorly, 4–6 rhoptries and coiled, multilamellar structures (Fig. 9). Although much of the host cell material was consumed by the developing parasite or used up in normal host cell metabolic activity, the nucleus appeared viable with its double membrane intact and chromatin dispersed in small peripheral blocks just under the nuclear membrane. Some host cell mitochondria and an endoplasmic reticulum remained around and between mature sporozoites. Even in acini with more than one infected cell there did not appear to be any noticeable host cell or nuclear hypertrophy. In several host cells, mature sporozoites, developing sporonts and recently penetrated kinetes were found, also without a noticeable increase in the size of the host cell (Fig. 10). Apparently not all parasitic stages developed to maturity as degenerating stages were found in a number of acini in which viable stages were found in adjacent acinar cells.

DISCUSSION

The sporogonic cycle of *B. occultans* in the salivary glands of *H. m. rufipes* begins when kinetes enter the secretory cells of acini. Similar kinetes found in the salivary glands of various ticks are more specifically referred to as sporokinetes in other *Babesia* spp., as they are derived from the asexual division of an ookinete (Mehlhorn & Schein, 1984; Young & Morzaria, 1986). It is likely that these asexual divisions also occur in *B. occultans* since newly invaded forms were found in the salivary glands throughout the course of tick feeding. Until this is confirmed, however, these initial stages are referred to as kinetes.

The process of asexual replication observed in the polymorphous sporonts of *B. occultans* conforms to the ultrastructural description of schizogony as reviewed by Aikawa & Sterling (1974). After repeated nuclear divisions, sporozoites form along the plasmalemma of the parasite mass at a point where newly formed segments of inner osmiophilic membrane have appeared. This is similar to the basic pattern of sporozoite formation described for several other *Babesia* spp. (Mehlhorn & Schein, 1984). However, when comparing specific developmental and morphological features of *B. occultans* to those described for other *Babesia* spp., variations were encountered which distinguish *B. occultans* from these other species.

The basic pattern of development of *Babesia equi* and *Babesia microti* in the salivary glands of their tick vectors is very similar to that observed in *B. occultans*. The kinetes of *B. equi* enter and begin development in the glands prior to tick feeding and the sporont develops into a massive meshwork with extensive cytoplasmic branching (Moltmann *et al.*, 1983). Sporonts of *B. microti* also develop into a large meshwork and sporozoites form
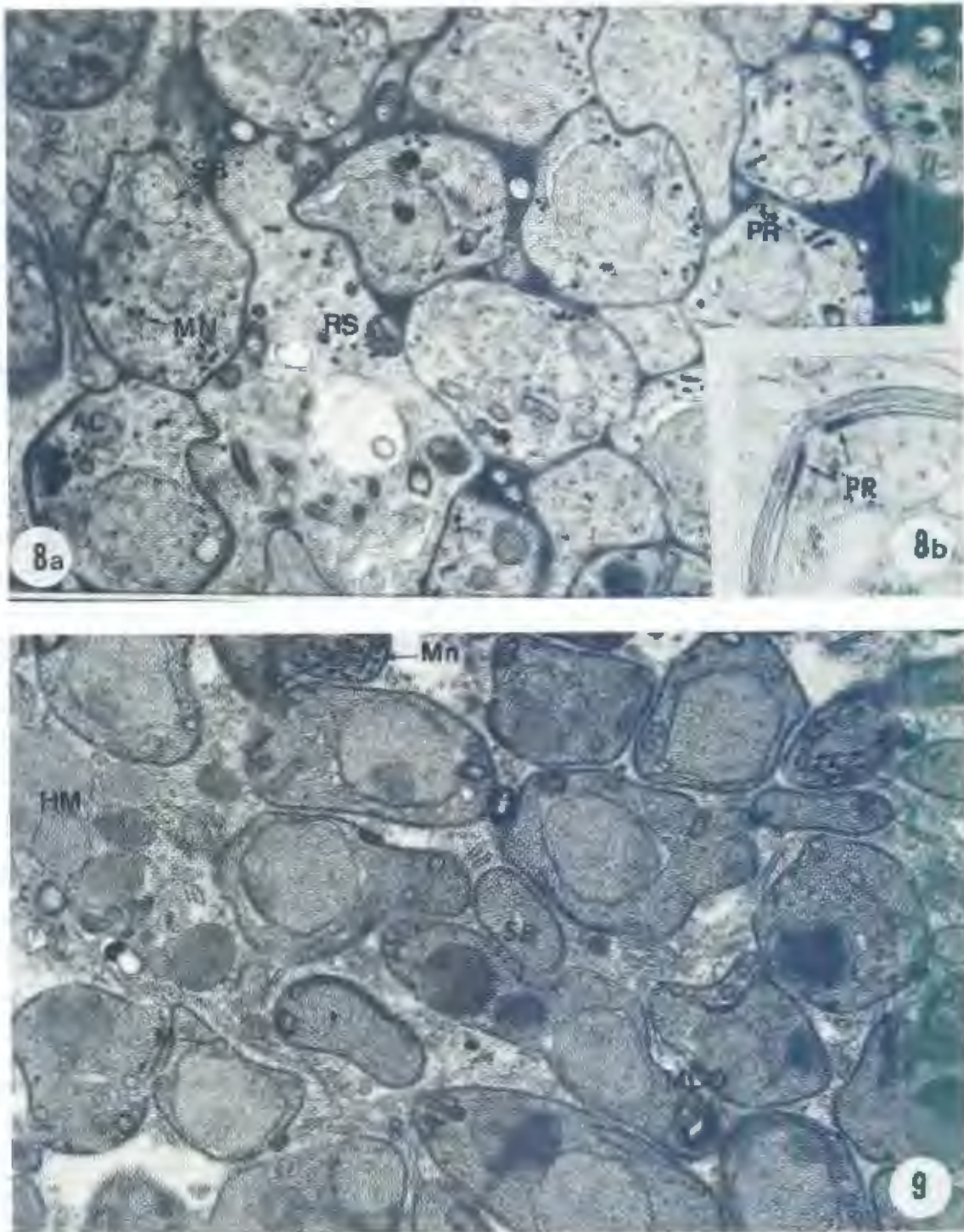
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FIG. 8–9 Electron micrographs of developmental stages of *Babesia occultans* in the salivary glands of *Hyalomma marginatum rufipes*

8 Cross-section through newly formed sporozoites still embedded within residual sporont cytoplasm (RS).

   a   Micronemes (Mn), polar rings (PR) and spherical bodies (SB) were observed in most sporozoites at this stage, while an apical complex (AC) was observed in a few. × 8 000

   b   A polar ring (PR) formed from the 2 membranes of the osmiophilic layer. × 45 000

9 Cross-section through mature sporozoites within remnants of the host cell matrix (HM). Rhoptries (R) and anteriorly concentrated micronemes (MN) can be seen in longitudinal sections of sporozoites. Numerous mitochondria (M) are present as well as multilamellar, coiled organelles. Note the 2 spherical bodies (SB) in 1 sporozoite. × 9 000

from the periphery of the mass but only after a simultaneous nuclear and cytoplasmic division in what Karakashian, Rudzinska, Spielman, Lewengrub, Piesman & Shourkrey (1983) refer to as true budding rather than schizogony. No evidence of a branching network was observed during the development of *B. occultans*. The extensive meshwork observed in these 2 other species is actually more characteristic of *Theileria* spp. (Fawcett, Young & Leitch, 1985) and by virtue of their additional reproduction in vertebrate lymphocytes may eventually be removed from the genus *Babesia* (Mehlhorn & Schein, 1984).
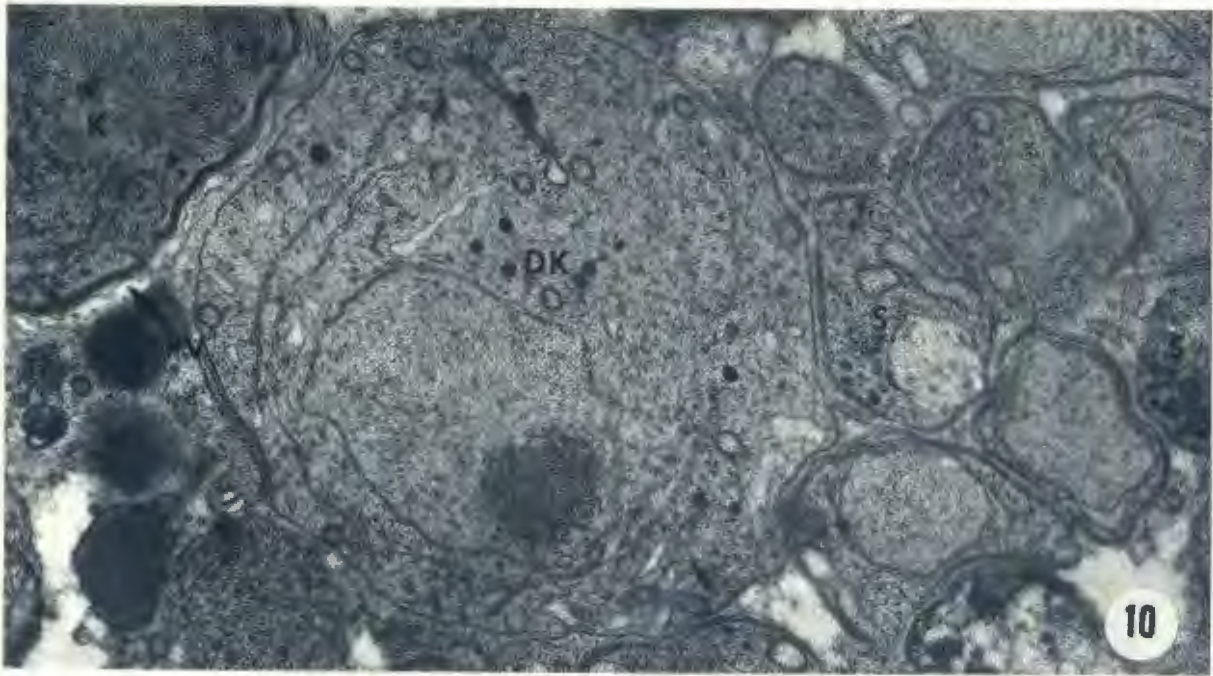
FIG. 10 Cross-section through a multiple infected granular acinar cell of *Hyalomma marginatum rufipes* containing several different developmental stages of *Babesia occultans*. Note sporozoites (S), differentiating kinetes (DK) and newly penetrated kinetes (K) still with a parasitophorous vacuole (PV). × 12 000

Of the other species in which development in the salivary glands of ticks has been investigated at the ultrastructural level, *Babesia ovis* has been shown to undergo schizogony, but cytomeres are formed prior to the differentiation of sporozoites (Moltmann, Mehlhorn & Schein, 1982). *Babesia canis* undergoes a distinct sequence of development among piroplasms in that sporozoites are formed as the terminal phase of a series of binary fissions (Schein, Mehlhorn & Voigt, 1979). The ultrastructure of both *B. bovis* and *B. bigemina* in tick salivary glands has also been investigated (Potgieter & Els, 1976; 1977), but the sequence of development leading to sporozoite formation was not determined. Large schizonts and significant host cell distention however, were reported in both species.

In comparing specific morphological features of *B. occultans* with those of other *Babesia* spp. the size of sporozoites of *B. occultans* (up to 3,5 μm in length), makes them one of the largest species described. The presence of a parasitophorous vacuole, spherical bodies and multilamellar organelles are characteristics which tend to be variable amongst the species. In the present study the presence of kinetes within a parasitophorous vacuole throughout tick feeding indicates that organisms are continually entering salivary glands from another site in the tick. The function or significance of spherical bodies has not been determined. Friedhoff (1981) speculated that the microneme-like structures found in these bodies may function in the rapid formation of membranes and microtubules in both sporozoites and merozoites. Their inconsistent presence among species, however, does make their significance difficult to determine.

Multilamellar structures are identified in many different parasitic protozoa (Scholtyseck, 1979). Friedhoff (1981) indicated that mitochondria of kinetes of *B. bigemina* are frequently bizarre in shape and contain membrane whorls. It may be that some of these multilammelar structures are just an aberrant form of an organelle such as a mitochondrion. Rudzinska (1981) suggested that these organelles might function in digestion and

waste removal, when peripherally located coiled organelles uncoil and rupture the plasma membrane of the parasite. In this study, many of the coiled organelles were found peripherally and in some cases appeared continuous with the parasite membrane (Fig. 2 & 9).

The results of this study have shown that sporozoites of *B. occultans* undergo a process of multiple fission which can be referred to as schizogony similar to that described for several other *Babesia* and *Theileria* spp. However, the extensive enlargement of the parasite mass and host cell hypertrophy is not seen. While this appears to be a distinctive feature of *B. occultans*, it may not be unique. In a light microscopic study of *B. argentina*, Reik (1966) revealed that schizonts in the salivary glands of *Boophilus microplus* were quite small. This observation needs to be investigated at the ultrastructural level.

The invasion and development of kinetes of *B. occultans* in previously infected acinar cells has not been reported in other species. Degenerating forms were identified in ticks at later stages of feeding along with viable forms and it may be that as the host cell cytoplasm is depleted there is not sufficient material to allow 2 or more kinetes to develop into mature sporozoites.

In summary, it is felt that the developmental and morphologic features of sporogony in the salivary glands of *H. m. rufipes* described in this study distinguish *B. occultans* from the other *Babesia* spp. which have been studied at the ultrastructural level. The actual process of sporozoite formation has been studied in only a few other species. Two of these (*B. equi* and *B. microti*) are thought to be more related to *Theileria* spp. and another (*B. canis*) undergoes a development pattern quite distinct among other piroplasms. Sufficient variation was encountered between *B. occultans* and the other *Babesia* spp. to warrant the investigation of sporogony in additional species to determine if there is a consistent pattern.

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