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THE FINE STRUCTURE OF MEROZOITES OF *BABESIA BOVIS* IN THE GUT EPITHELIUM OF *BOOPHILUS MICROPLUS*

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ABSTRACT

POTGIETER, F. T., ELS, H. J. & VAN VUUREN, A. S., 1976. The fine structure of merozoites of *Babesia bovis* in the tick vector *Boophilus microplus*. *Onderstepoort J. vet. Res.*, 43 (1), 1–10 (1976).

Electron microscopic studies on merozoites of *Babesia bovis* in epithelial cells of the gut of *Boophilus microplus* revealed that the pellicle apparently consists of 3 membranes, and an osmiophilic layer intimately associated with microtubules. Micropores in the pellicle were often associated with micronemes. An unidentified tubular structure extended from the anterior polar ring to the nuclear region where it appeared to be associated with the nuclear envelope. A Golgi complex, typical protozoan mitochondria, food vacuoles and rhoptries could not be identified.

Résumé

POTGIETER, F. T., ELS, H. J. & VAN VUUREN, A. S., 1976. *La structure en microscopie électronique des mérozoites de* Babesia bovis *dans l'epithélium intestinal de la tique* Boophilus microplus. Onderstepoort J. vet. Res. 43 (1), 1–10 (1976).

D'après des études en microscopie électronique effectuées sur mérozoïtes de Babesia bovis *dans l'épithélium intestinal du tique* Boophilus microplus, *la pellicule paraît consister de 3 membranes en plus d'une couche osmiophilique étroitement associée aux microtubules. Des micropores dans la pellicule sont souvent liés avec des micronèmes. Une structure tubulaire non encore identifiée passe de l'anneau polaire antérieur jusqu'à la région nucleaire, avec l'enveloppe nucléaire de laquelle elle paraît être liée. Ni un appareil de golgi, ni des mitochondries protozoaires typiques, ni des vacuoles nourricières, ni des rhoptries n'ont pu être identifiés.*

INTRODUCTION

Trophozoites, fission bodies and merozoites (=vermicules) are the only developmental stages of the babesias which have been identified. Electron microscopic studies (Friedhoff & Scholtyseck, 1968a; 1968b; 1969; Scholtyseck, Friedhoff & Piekarski, 1970; Scholtyseck & Mehlhorn, 1970; Scholtyseck, Mehlhorn & Friedhoff, 1970) have revealed the fine structure of the apical complex in members of the family Babesiidae Poche (1913). These observations include the presence of organelles such as polar rings, rhoptries, micronemes, microtubules and micropores. No conoid has been identified with certainty in any piroplasm. Certain ultrastructural characteristics have since been used as taxonomic criteria for these organisms (Levine, 1971). This study on the fine structure of merozoites of *Babesia bovis* (Babés, 1888) in the gut epithelial cells of *Boophilus microplus* (Canestrini, 1888) revealed certain features of the merozoites which have not been described before.

MATERIALS AND METHODS

Larvae of a non-infected laboratory strain of *Boophilus microplus* were used to infest 2 susceptible splenectomized oxen. One animal subsequently received *Babesia bovis* infected larvae of the same tick strain on day 12 after the primary infestation, while the other acted as an uninfected control.

Engorged female ticks commenced to drop from both animals on day 20. *B. bovis* parasites were also observed for the first time on day 20 in a thick blood smear of the animal that received infected ticks. On day 23 this animal had a *B. bovis* parasitaemia of 2,1% and all ticks that had engorged during the preceding 24 hours were collected and kept at 25 °C and 90% relative humidity. Seven days after dropping, haemolymph smears were made from these ticks stained with Giemsa, and examined with a light microscope for the presence of merozoites of *B. bovis*. Heavily infected ticks were dissected and parts of the gut removed for electron microscopic examination. The control animal did not contract babesiosis and no evidence of a *B. bovis* infection could be detected in haemolymph smears of ticks collected from this animal.

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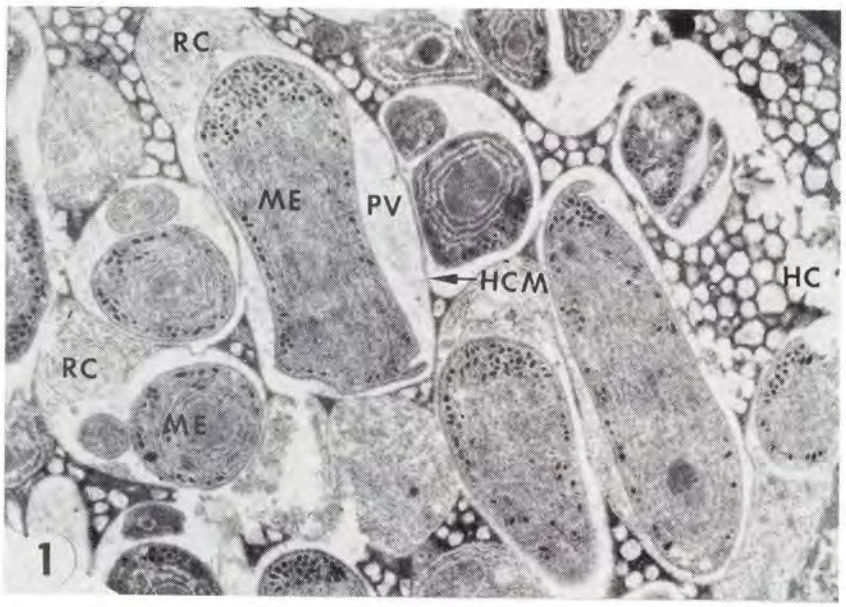
The dissected gut specimens were immediately fixed in a 2,5% solution of glutaraldehyde buffered with 0,05 M sodium cacodylate (pH 7,2) for 1 hour at 4 °C. After fixation the specimens were washed twice for 15 min in sodium cacodylate buffer and were subsequently post-fixed in a 1% solution of osmium tetroxide for 1 hour, washed in Millonig's buffer (Millonig, 1961) and dehydrated through a graded series of ethanol concentrations at 4 °C. The concentrations used were 30%, 50%, 70% 95% and 100%. Dehydration was allowed to proceed for 15 min at each concentration and repeated 3 times in 100% ethanol. The specimens were cleared in two 15 min washes in propylene oxide, then placed in a 1:1 mixture of porpylene oxide and Epon embedding medium for 1–12 hours before embedding in Epon. The blocks were shaped and sectioned on a Reichert Om U_2 ultramicrotome. The sections were stained with a 5% aqueous solution of uranyl acetate, freshly prepared before use, washed in distilled water and counter stained in lead citrate. The sections ranged from 60–90 nm in thickness and were viewed in a Siemens Elmiskop 1A electron microscope.

RESULTS

Only the merozoites of *B. bovis* in *B. microplus* 7 days after repletion were examined during the course of this study. These merozoites were found intracellularly in gut epithelial cells of the engorged female ticks.

Two stages of the merozoite were identified, viz. multifolded forms with rounded outlines and typical vermicule shaped types. The folded forms ranged from spherical to ovoidal in shape (Fig. 2 & 17) while the vermicules were broad anteriorly and tapered gradually from the nuclear region to the posterior end (Fig. 5a & 18a). The anterior pole protruded slightly in the case of the vermicules. During the course of this study it became apparent that, apart from shape, very few ultrastructural differences existed between the 2 forms. The characteristics of organelles seen in both will therefore be described concomitantly. The folded spherical forms will be referred to as immature merozoites and the unfolded vermicules as mature merozoites.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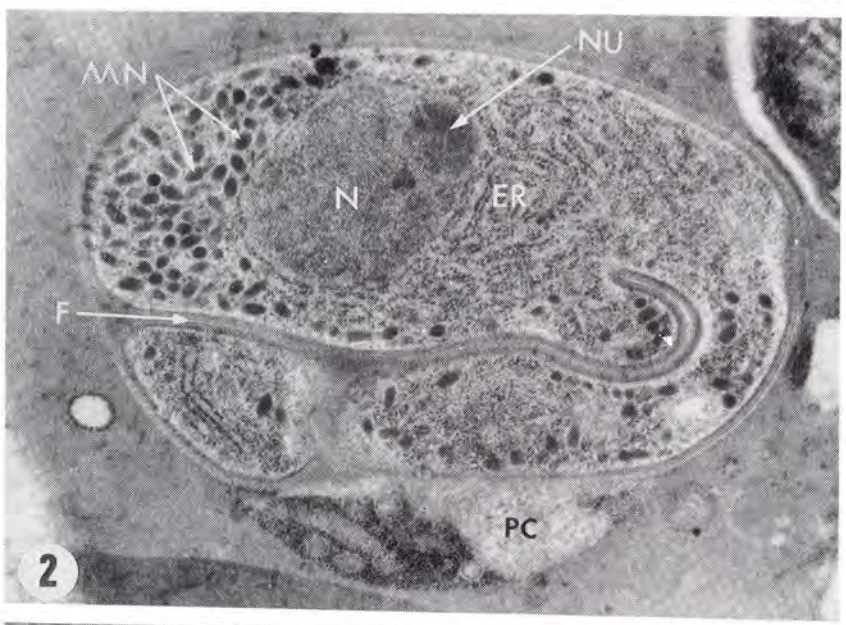
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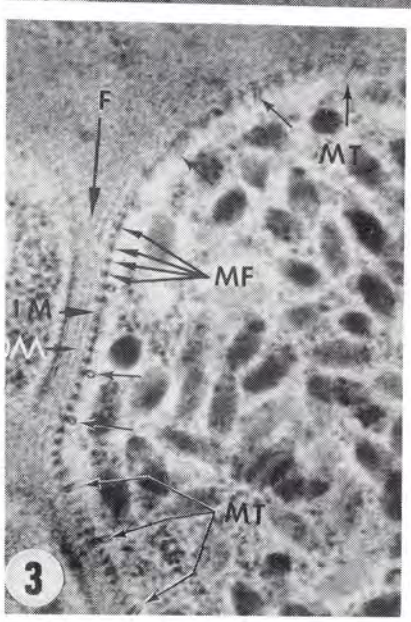
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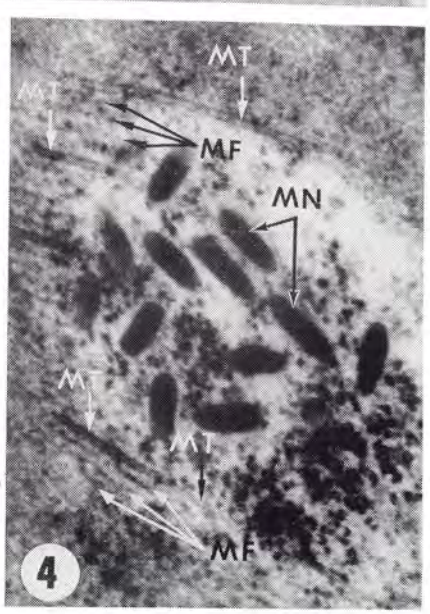
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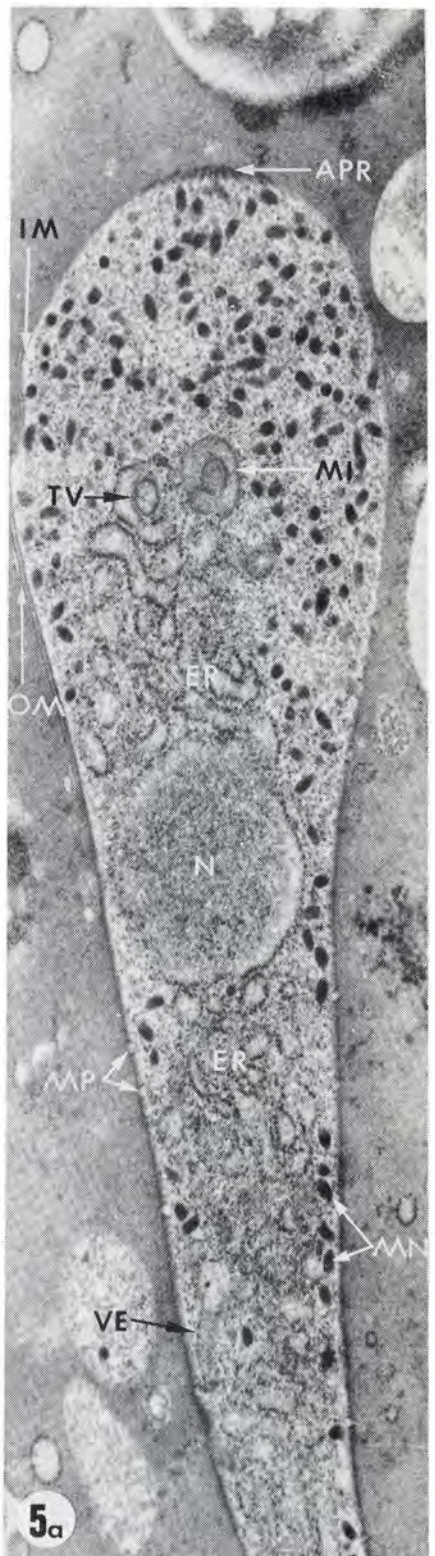
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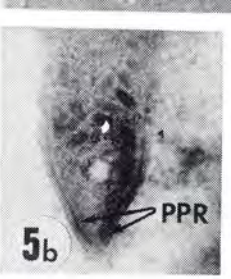
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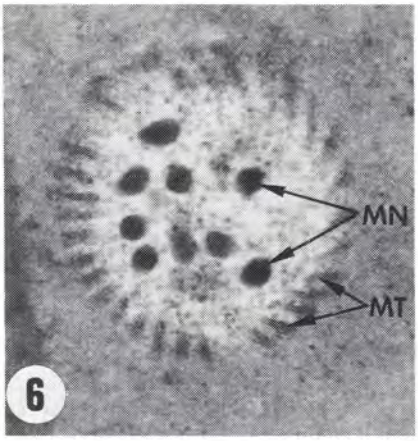
FIG. 1–18 Electron micrographs of *B. bovis* merozoites in gut epithelial cells of *B. microplus*
FIG. 1 Low magnification view of immature merozoites and residual cytoplasm of parasitic origin in parasitophorous vacuoles. ×7 000
FIG. 2 Longitudinal section of a folded spherical form (immature merozoite); note parasite cytoplasm between inner and outer membranes of the pellicle. ×21 000
FIG. 3 Cross section of immature merozoite. Note arrangement of microfibrils in close association with inner membranes of the parasite pellicle and pellicular microtubules. ×52 000
FIG. 4 Tangential section through part of pellicle of mature merozoite. Microtubules and microfibrils are visible. ×52 000
FIG. 5a Longitudinal section through a mature merozoite. ×21 000
FIG. 5b Longitudinal section of posterior end of mature merozoite. Posterior polar ring is clearly visible. ×21 000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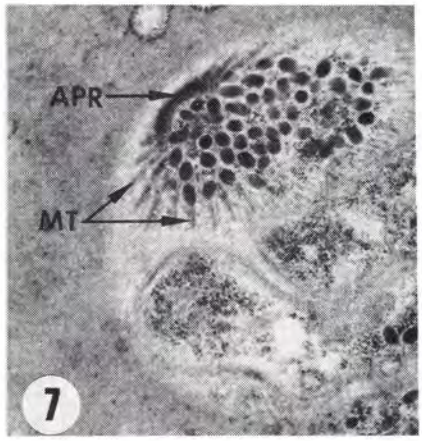
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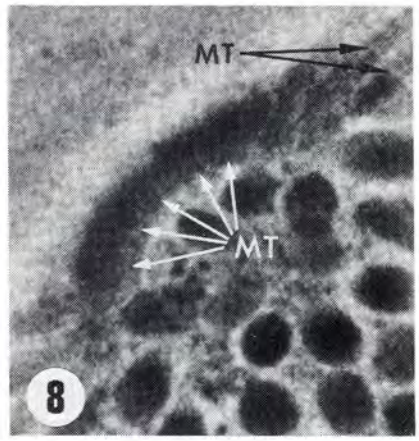
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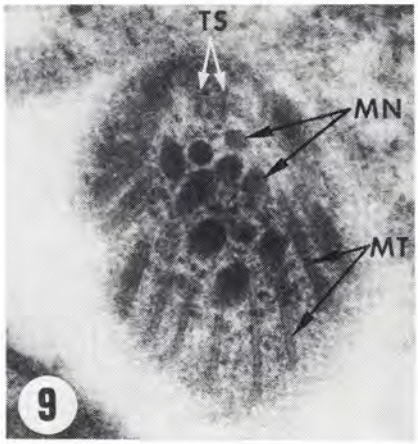
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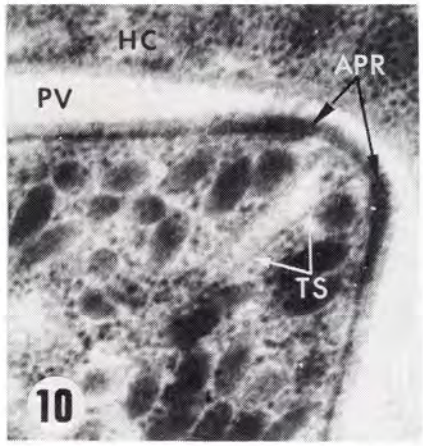
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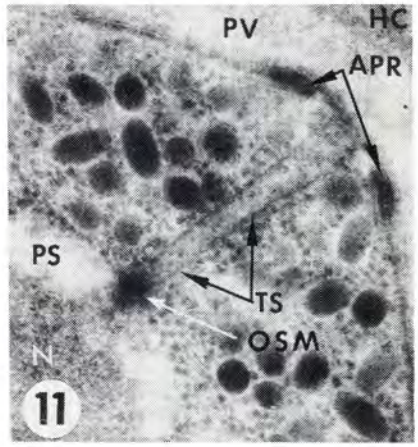
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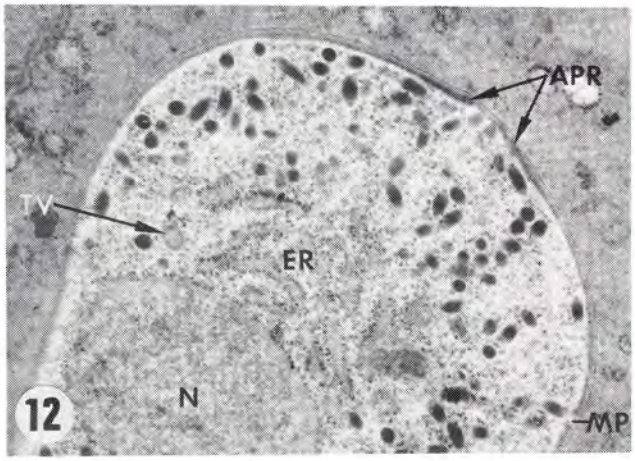
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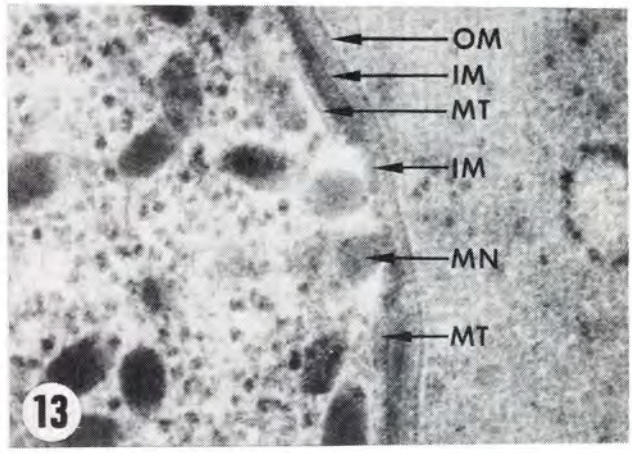
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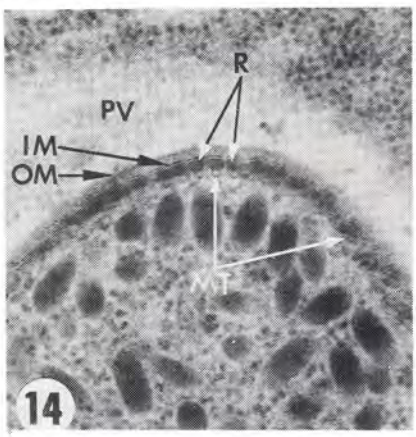
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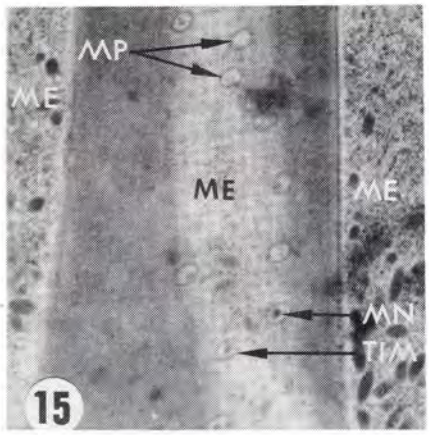
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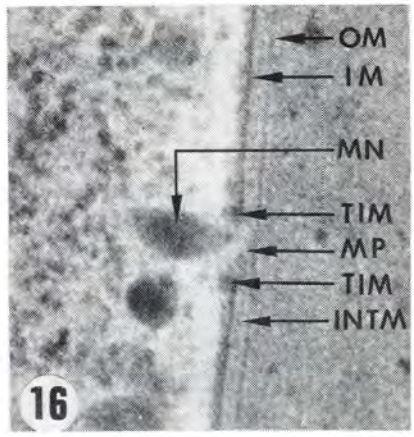
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FIG. 6 Oblique section through mature merozoite near anterior end. Note the arrangement of microtubules. ×70 000
FIG. 7 Tangential section through the anterior polar complex of folded spherical form. Note part of polar ring and associated microtubules and microfibrils. ×21 000
FIG. 8 Part of organism in Fig. 7, photographed at higher magnification showing microtubules forming polar ring. ×84 000
FIG. 9 Tangential section through anterior polar ring of a folded spherical form in parasitophorous vacuole. Note tubular structure seen in Fig. 10 & 11 extending into the centre of the polar ring. ×52 000
FIG. 10 Longitudinal section through a folded spherical merozoite in a parasitophorous vacuole. Note the tubular structure extending from the centre of the polar ring in the direction of the nucleus. ×52 000
FIG. 11 Longitudinal section of anterior part of folded spherical form in parasitophorous vacuole. Tubular structure similar to that in Fig. 10, can be seen extending from the centre of the anterior polar ring to the nuclear envelope. ×52 000
FIG. 12 Longitudinal section through the anterior end of mature merozoite. ×25 000
FIG. 13 High magnification of the anterior polar ring of organism in Fig. 12. ×77 000
FIG. 14 Tangential section through the polar region of folded spherical form. The microtubules are in close association with the osmiophilic "ribs" near the anterior polar ring. ×52 000
FIG. 15 Surface view of micropores in inner membrane of pellicle. Note circular rings around micropores. ×21 000
FIG. 16 Longitudinal section of micropore in inner membrane of a mature merozoite. Note the close association between a microneme and this organelle. ×84 000


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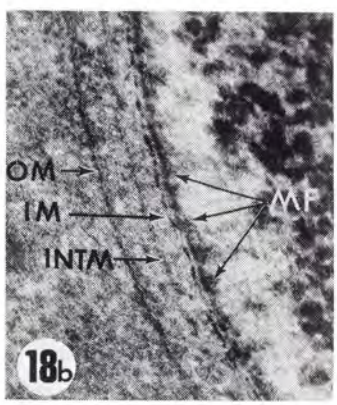
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Location of merozoites

Many of the immature merozoites were located in parasitophorous vacuoles (Fig. 1, 9, 10 & 11). Some of these vacuoles were observed to contain fine granular material and traces of degenerating endoplasmic reticulum apparently of parasitic origin (Fig. 1). Other immature forms were in direct contact with host cell cytoplasm and appeared to have some cytoplasm between their inner and outer pellicular membranes (Fig. 2 & 17). All the mature merozoites were in direct contact with the host cell cytoplasm, and no residues were observed to be associated with them (Fig. 5a & 18a). No extracellular merozoites were encountered.

Pellicle of the parasite

Pellicular membranes. Merozoites in direct contact with the host cell cytoplasm showed evidence of 3 membranes (Fig. 16, 17 & 18b). The outer membrane appeared as a single layer followed by a poorly staining intermediate middle membrane. The inner membrane had a granulated osmiophilic appearance and appeared to have a unit membrane structure. The inner layer, however, was so intimately associated with the underlying microfibrillar layer (see below) that this could not be confirmed (Fig. 3, 4, 14 & 18b). Under low magnification only the inner and outer membranes were discernible. Unfortunately none of the parasites observed in vacuoles proved to be suitable for a high resolution study of the pellicular structure (Fig. 1, 10, 11 & 14).

Osmiophilic layer. A layer of electron dense material was located below the inner membrane and intimately associated with it. It extended across the length of the body of the merozoite. In cross sections of the mid region of the merozoite, this layer consisted of numerous regularly spaced longitudinal microfibrils lying between the microtubules (Fig. 3 & 4). Near the tapering anterior end, however, these microfibrils converged and were seen as 32 relatively thick osmiophilic "ribs" (Fig. 14). At the anterior, and to a lesser extent the posterior pole, the congregated electron dense material became amorphous and appeared to form a major component of the polar rings (Fig. 7. 8 & 5b).

Pellicular microtubules. Longitudinally arranged microtubules were observed underneath the inner membrane of the pellicle and in close association with the osmiophilic layer (Fig. 3, 4, 6, 7, 8 & 9). Near the anterior pole 32 microtubules were located immediately below the electron dense "ribs", and partly embedded in them (Fig. 14). The microtubules originated in the anterior polar ring and extended along the body wall of the merozoite. Tubules were seen in cross sections of the parasite as far down as the nuclear region, but the exact distance that they extended towards the posterior pole could not be determined.

Polar rings

The anterior polar ring consisted of converging microtubules associated with, and supported by highly concentrated amorphous electron dense material (Fig. 7, 8, 9). No individual mecrofibrils or "ribs" could be distinguished in the actual polar ring (Fig. 7 & 8). In the ring the ends of the microtubules folded back upon themselves for a short distance (Fig. 13). Both the outer and inner membranes extended across the polar ring (Fig. 12 & 13).

The posterior polar ring is formed by the aggregation of osmiophilic material directly below the inner membrane (Fig. 5b). It is less prominent than the anterior polar ring, and no evidence was obtained on possible involvement of the microtubules in the composition of this ring.

Micropores

Micropores were observed in the inner membrane but showed no fixed pattern of distribution (Fig. 5a, 12, 15 & 16). The walls of these pores appeared as thickenings of the inner membrane and especially the underlying osmiophilic layer (Fig. 16). These thickenings formed circular rings clearly visible in cross section (Fig. 15). Micronemes were often seen in close association with micropores. Some of these micronemes were located partially within the micropores, while others appeared to be directed towards it (Fig. 15 & 16).

Tubular structure

In a number of immature forms located in parasitophorous vacuoles, a tubular structure was seen extending from the nuclear envelope to the anterior polar ring (Fig. 9, 10, & 11). In Fig. 9, the wide end of this tapering organelle can be seen inside the anterior polar ring. The narrow end is joined to the nuclear envelope by osmiophilic material (Fig. 11). No direct association could be detected between this structure and the endoplasmic reticulum or micronemes.

Nucleus

The nucleus is spherical in shape and situated in the anterior half of the body of the merozoite (Fig. 5a & 18a). It is enveloped by a double membrane (Fig. 18a) and in most cases the perinuclear space is prominent (Fig. 17 & 18a). A well defined nucleolus was observed in some organisms (Fig. 2). The folded form in Fig. 17 apparently has two nuclei, but this may be the result of a sectioning artefact. No other multinucleated merozoites were seen.

Other organelles

A rough type of endoplasmic reticulum was present in the cytoplasm surrounding and posterior to the nucleus, but was absent from the regions where micronemes were concentrated (Fig. 5a & 18a). The micronemes were found in the area between the nucleus and anterior polar ring, as well as along the body wall and at the posterior pole (Fig. 5a, 5b & 18a).

Small thick-walled vesicles were observed, some with the contents staining more intensely than the ground substance of the cytoplasm (Fig. 12, 17 & 18a). A larger type of vesicle was observed anterior to the nucleus in mature merozoites. The latter vesicles were surrounded by osmiophilic membranes, and some had a concentric membrane in the centre (Fig. 5a, & 18a). These were the only structures remotely resembling mitochondria.

FIG. 17 Longitudinal section through a folded merozoite. Note the presence of 3 limiting membranes and parasite cytoplasm between the inner and outer membranes. (This may be a binucleate form). ×34 000
FIG. 18a Longitudinal section through a mature merozoite. ×43 000
FIG. 18b High magnification of the pellicle of merozoite seen in Fig. 17. ×100 000

A Golgi complex, typical protozoan mitochondria, food vacuoles and rhoptries could not be identified in this study.

Discussion

The fine structure of developmental stages of *B. ovis* and *B. bigemina* in ticks has been studied previously, (Friedhoff & Scholtyseck, 1968a, 1968b; and Friedhoff & Scholtyseck, 1969). This is the first report, however, on the ultrastructure of a stage in the life cycle of *B. bovis* in the tick vector. The merozoites of *B. bovis* described here resemble those of the other 2 species in many respects, but some notable differences became evident during the course of this study.

Studies on *B. ovis* (Friedhoff & Scholtyseck, 1968b; Scholtyseck, *et al.*, 1970) and *B. bigemina* (Friedhoff & Scholtyseck, 1969), revealed that some stages occurred in parasitophorous vacuoles, while others were in direct contact with the host cell cytoplasm. The spherical forms of *B. bigemina* were located in vacuoles surrounded by more than one membrane. These membranes were found to be continuous with the endoplasmic reticulum of the host cell. Friedhoff & Scholtyseck (1968a) reported that the intracellular spherical forms of *B. ovis* are surrounded by 2 membranes of which the outer one is derived from the host cell cytoplasm. In the present study no association could be detected between the membranes which surrounded the parasitophorous vacuoles, the endoplasmic reticulum of the host cell cytoplasm, or the pellicle of the parasite. Although conclusive evidence is lacking it is thought that in *B. bovis* infections, these membranes originate from the host cell cytoplasm.

Unfolded merozoites of *B. bigemina* (Friedhoff & Scholtyseck 1969) and certain developing forms of *B. ovis* (Scholtyseck *et al.*, 1970) have been observed to be in direct contact with the host cell cytoplasm. Some of the immature merozoites of *B. bovis* and all the mature ones were similarly located.

Friedhoff & Scholtyseck (1968a) recorded the presence of "Cytoplasmareste" between the two membranes which surrounded developing forms of *B. ovis*, and "Cytoplasmaanteile" between the pellicle of the parasite and the membranes of the parasitophorous vacuoles. In addition, Scholtyseck *et al.* (1970) found "Restcytoplasma" between the inner and outer membranes of developing forms of *B. ovis* which were in direct contact with the host cell cytoplasm. Similar structures have not been described for *B. bigemina*. Nothing resembling the "Cytoplasmareste" of *B. ovis* was observed in this study. Finely granular material and traces of endoplasmic reticulum were in the parasitophorous vacuoles of some of the immature merozoites of *B. bovis*. In location these resembled the "Cytoplasmaanteile" of *B. ovis*. The cytoplasm present between the inner and outer membranes of the pellicle, of those immature merozoites of *B. bovis*, in direct contact with the host cell cytoplasm, can possibly be related to the "Restcytoplasma" found in *B. ovis*.

The absence of cytoplasm between the inner and outer membranes of the mature merozoite may indicate that it becomes incorporated in the cytoplasm of the merozoite prior to unfolding, or discarded during the process.

The pellicle of *B. ovis* and *B. bigemina* consists of an outer and a thicker inner membrane (Friedhoff & Scholtyseck 1968a; 1968b; 1969; Scholtyseck *et al.*, 1970). In some developing forms of *B. ovis* Scholtyseck & Mehlhorn (1970) found that the thick inner layer of the pellicle consisted of two unit membranes. High resolution microscopy of *B. bovis* merozoites in direct contact with host cell cytoplasm, showed the presence of a poorly staining, interrupted, intermediate membrane between the outer and inner membranes. The latter consisted of an osmiophilic granular membrane intimately associated with an underlying osmiophilic layer.

According to Andreassen & Behnke (1968), the term "pellicle" by definition comprises the plasma membrane and the peripheral "solid" part of the cytoplasm. The pellicle of *B. bovis* would then be formed by the 3 membranes, the osmiophilic microfibrillar layer and the microtubules arising from the anterior polar ring. Pitelka (1963) also considers the microtubules to be part of the pellicle.

Friedhoff & Scholtyseck (1969) described 32 elevations of the inner membrane "ribs" which arise in the apical region of the merozoites of *B. bigemina*. These "ribs" increased in thickness as they converged on the polar ring, and appeared as thick-walled tubes closely associated with microtubules arising from the polar ring. The same structure was apparently found in merozoites of *B. ovis* (Scholtyseck, *et al.*, 1970). In *B. bovis* merozoites, however, the microfibrils below the inner membrane became gradually more closely arranged, thicker and stained more intensely towards the anterior region where it appeared as solid osmiophilic "ribs". No thick-walled tubes were seen, but microtubules were present immediately below the "ribs" and were often partly embedded in osmiophilic material. The thick-walled tubes described for *B. bigemina* and *B. ovis*, appear to occupy the same position as the microtubules of merozoites of *B. bovis*, i.e. in the vicinity of the anterior polar ring. The number of "ribs" in *B. bigemina* (Scholtyseck, *et al.*, 1970) also coincided with the number of microtubules seen in *B. bovis*, viz. 32.

The micropores of merozoites of *B. bovis* are different from those described for *B. ovis* (Friedhoff & Scholtyseck, 1968a; Scholtyseck & Mehlhorn, 1970). In *B. ovis* both the outer and inner membranes take part in the formation of the micropores and these organelles apparently behave as cytostomes. Only the inner membrane and the associated microfibrillar layer are involved in the formation of *B. bovis* micropores, whereas the outer membrane is not affected. The close association between the micronemes and micropores in merozoites of *B. bovis* may indicate a secretory or excretory function of the latter, depending on the actual function of the micronemes which is as yet not fully understood. No micropores have been reported for *B. bigemina*.

The function of the tubular structure connecting the nuclear envelope with the apical complex of immature merozoites of *B. bovis* is unkown. Its extension into the polar ring may well indicate an excretory function or possibly secretion during cell penetration. The latter seems unlikely, however, as this structure was not observed in mature merozoites where such a mechanism could be of importance. The initimate electron dense junction between the tapering end of the tube and the outer membrane of the nuclear envelope appears to suggest some activity involving the nucleus and associated granular endoplasmic reticulum.

Certain basic similarities exist in the fine structure of the *Babesia* spp. discussed. The important differences which occur in the merozoites of *B. bovis* described here, such as the structure of the pellicle and the presence of micropores and a tubular structure, indicate that further investigation would be of value.

List of Abbreviations used in all Micrographs

APR—Anterior polar ring
ER—Endoplasmic reticulum
F—Fold in parasite pellicle
HC—Host cell
HCM—Host cell membrane
IM—Inner membrane
INTM—Intermediate membrane
ME—Merozoite
MF—Microfibril
MI—Mitochondrion (?)
MN—Microneme
MP—Micropore (in inner membrane)
MT—Pellicular microtubule
N—Nucleus
NM—nuclear membrane
NU—Nucleolus
OM—Outer membrane
OSM—Osmiophilic material
PC—Parasite cytoplasm
PPR—Posterior polar ring
PS—Perinuclear space
PV—Parasitophorous vacuole
R—Ribs
RC—Residual cytoplasm of parasitic origin
TIM—Thickening of inner membrane
TS—Tubular structure
TV—Thick-walled vesicle
VE—Vesicle

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