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COMPARATIVE ULTRASTRUCTURAL STUDIES ON *BESNOITIA BESNOITI* AND *BESNOITIA CAPRAE*

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ABSTRACT

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Comparative transmission electron microscopy on *Besnoitia besnoiti* and on a strain of *Besnoitia* derived from goats in Kenya revealed that the two organisms differ in their pellicle, micropore, microtubules, nucleus, wall-forming body 1 (W1), amount of lipids and amylopectin. Thus the caprine besnoitia is probably a different organism and the term *Besnoitia caprae* should continue to be used.

Keywords: *Besnoitia besnoiti*, *Besnoitia caprae*, cystozoite, goat, ultrastructure, speciation

INTRODUCTION

Preliminary findings on the infectivity of the strain of *Besnoitia* that infects goats in Kenya showed that rabbits, hamsters, guinea pigs, rats, mice and cattle were refractory to it. The organism therefore appeared to differ from *Besnoitia besnoiti*, which is infective for cattle and rabbits (Pols, 1960; Bigalke, 1967), mice (Kaggwa *et al.*, 1979) and hamsters (Bigalke, 1967) and was termed *Besnoitia caprae* (Njenga *et al.*, 1993).

The ultrastructure of cultured *B. besnoiti* has been described (Neuman, 1974; Gobel *et al.*, 1985; Shkap *et al.*, 1988). Shkap and colleagues (1988) also studied the ultrastructure of cystozoites from a naturally infected bull. All these reports showed that *B. besnoiti* was typical of the apicomplexa, with a conoid, polar ring, microtubules and rhoptries.

Heydorn and colleagues (1984) described the histological and ultrastructural appearance of cysts from the eyelid of goats in Kenya. They confirmed that the cysts belonged to the genus *Besnoitia*, and that the merozoites had the typical three-layered coccidian pellicle, 22 microtubules, conoid, polar ring, rhoptries, dense bodies, polysaccharide granules, tubular mitochondrion and micronemes. They stated that the ultrastructure of the cysts and merozoites closely resembled those of other *Besnoitia* species.

The ultrastructure of *B. caprae* was studied to ascertain whether there were any physical differences between it and *B. besnoiti*.

MATERIALS AND METHODS

Subcutaneous fascia from the necks of a goat and a bull, respectively, suffering from experimentally induced *Besnoitia* infections, were harvested from freshly killed animals 70 days postinoculation. The goat and bull had been inoculated with field isolates of *B. caprae* and *B. besnoiti*, respectively, using the protocol previously described (Njenga *et al.*, 1993) and confirming infection using the cysts in the eye method (Bigalke and Neude, 1962).

The fascia were cut into small pieces of 1 × 1 × 1 mm and processed as a batch by the method of Ito and Karnovsky (1968) as follows. The small pieces of fascia were fixed in 4% paraformaldehyde dyed with 0.01% trinitrocresol and 2% glutaldehyde for 2 h at 4°C. The tissues were then rinsed three times for 5 min each at 4°C in phosphate buffer, pH 7.2–7.4 and postfixed for 4 h at 4°C in 1% osmium tetroxide in the same buffer. Following three further rinses, they were dehydrated at 4°C through graded concentrations of acetone prior to embedding in epoxy resin (Durcupan ACM, Fluka Ag, Switzerland). The tissues were sectioned with an ultramicrotome (Reichert Austria OM U2) and the sections were mounted on copper grids, stained with uranyl acetate, counterstained with lead citrate and examined using a Carl Zeiss 109 electron microscope.

RESULTS

The cystozoites of both *B. caprae* and *B. besnoiti* were crescent-shaped and bound by a three-layered pellicle. However, the pellicle of *B. caprae* was smooth, while that of *B. besnoiti* was wavy, with the outer membrane appearing to form junctions between itself and the inner membrane. The outer membrane of the pellicle was continuous and enveloped the whole organism in both species. The inner membrane and the outer membrane were separated by an osmiophobic space, which was consistently wide in *B. caprae* but variable in *B. besnoiti*. The inner membrane was continuous over most parts of the organism, except where the outer membrane invaginated to form micropores and anteriorly, where it thickened and terminated at the same level as the anterior conoid (Figures 1, 2, 6 and 7).

The micropore was formed in both species by invagination of the outer membrane of the pellicle (Figures 1, 2, 6 and 7). The outer cylindrical structure of the micropore in *B. caprae* was formed from a thickening of the inner membrane of the pellicle and an outgrowth from the outer membrane of the pellicle at the point where invagination starts. In *B. besnoiti*, the cylindrical structure was formed solely by the thickening of the inner membrane of the pellicle, which began well before the point of invagination. In both species, the cylindrical structure lining the micropore terminated where the hemisphere of the micropore widened out fully (Figures 1, 3, 4, 6 and 7).

The anterior ends of both parasites were typically apicomplexan in nature. They had a conoid, ducts of rhoptries, microtubules, micronemes and a polar ring. The conoid was in both cases cone-shaped with fibrillar structures arranged in a spiral.

There were three pairs of ducts of rhoptries in both species, which originated from within the conoid. They were long club-like osmiophilic structures that stretched from the anterior end to the level of the nucleus (Figures 1, 2 and 6).

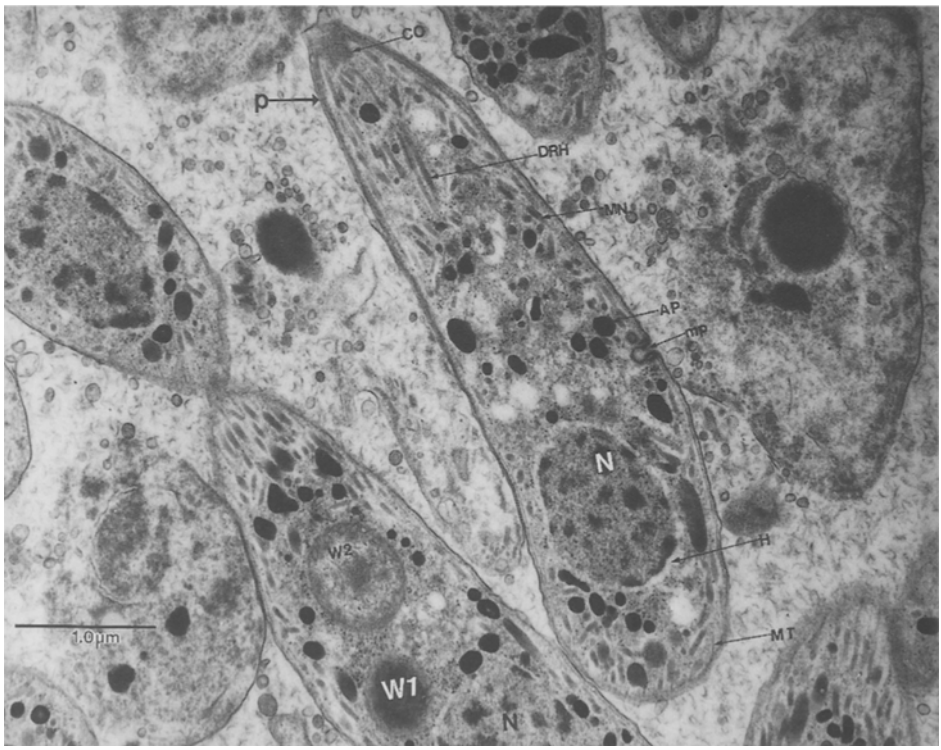

Figure 1. A micrograph of longitudinal and tangential sections of cystozoites of *Besnoitia caprae* showing the pellicle (P), the conoid (CO), the long club-like ducts of rhoptries (DRH), the short club-like micronemes (MN), the amylopectin granules (AP), the micropore (mp), the nucleus (N) surrounded by a halo zone (H), the microtubules (MT) and wall-forming bodies 1 (W1) and 2 (W2). Note the crescent shape of the complete organism, the smooth appearance of the pellicle, the spiral-fibrillar structure of the conoid, the osmiophilic nature of the ducts of rhoptries, micronemes and amylopectin, the parallel arrangement of the microtubules, the osmiophilic, dense homogenous nature of the W1 body and its closeness to the nucleus, and the spongy honeycomb internal appearance of the W2 body. The complete organism has neither the W1 body nor the W2 body (70 days after inoculation). (×18 200)

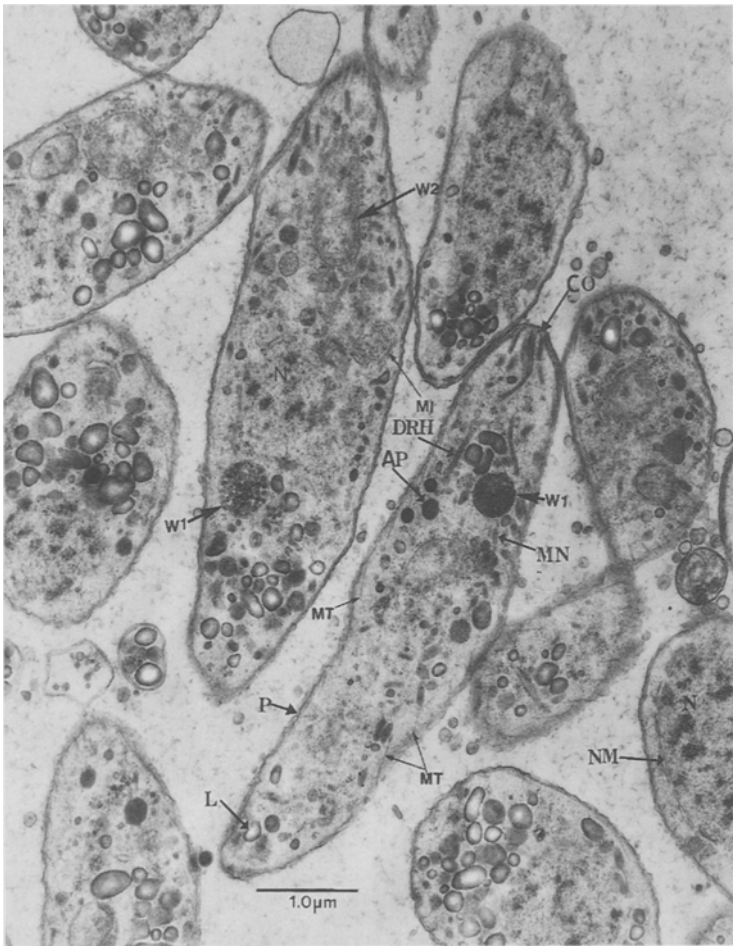

Figure 2. A micrograph of longitudinal and tangential sections of cystozoites of *Besnoitia besnoiti* showing the pellicle (P), the conoid (CO), the long club-like ducts of rhoptries (DRH), the short club-like micronemes (MN), the amylopectin (AP) and lipid granules (L), the mitochondrion (MI), the nucleus with and without the nuclear membrane (NM), the microtubules (MT) and wall-forming bodies 1 (W1) and 2 (W2). Note the crescent shape of the complete organism, the wavy appearance of the pellicle, the spiral-fibrillar structure of the conoid, the osmophilic nature of the ducts of rhoptries, micronemes, amylopectin, the lipid granules, and the oblique (spiral) arrangement of the microtubules. The W1 body is membrane-bound and has small membrane-bound osmiophilic dense bodies in it. The W2 body has a spongy internal appearance (70 days postinoculation) (×13 450).

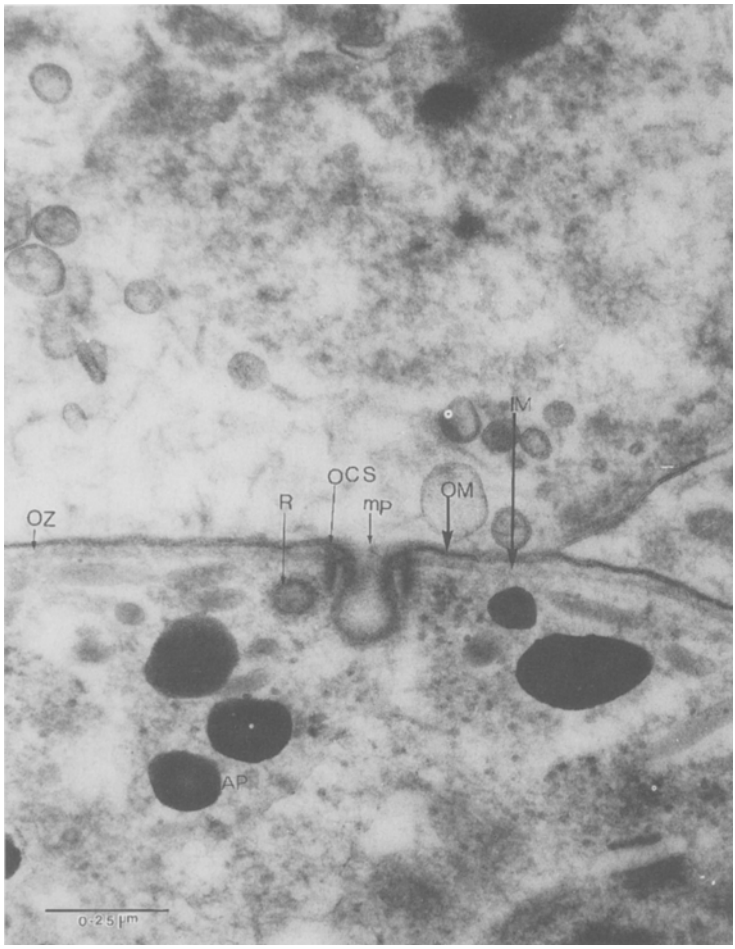

Figure 3. A micrograph of the fine details of a longitudinal section of the micropore (mp) of *Besnoitia caprae*. Note that the outer cylindrical structure (OCS) of the micropore appears to be formed from the outer (OM) and inner (IM) membranes of the pellicle. The osmiophobic zone (OZ) separating the two membranes is even. Ribosome-like structures (R) are found in the cytoplasm of the cystozoite and free in the cyst fluid. Amylopectin (AP) granules were also seen in this section. (×72 800)

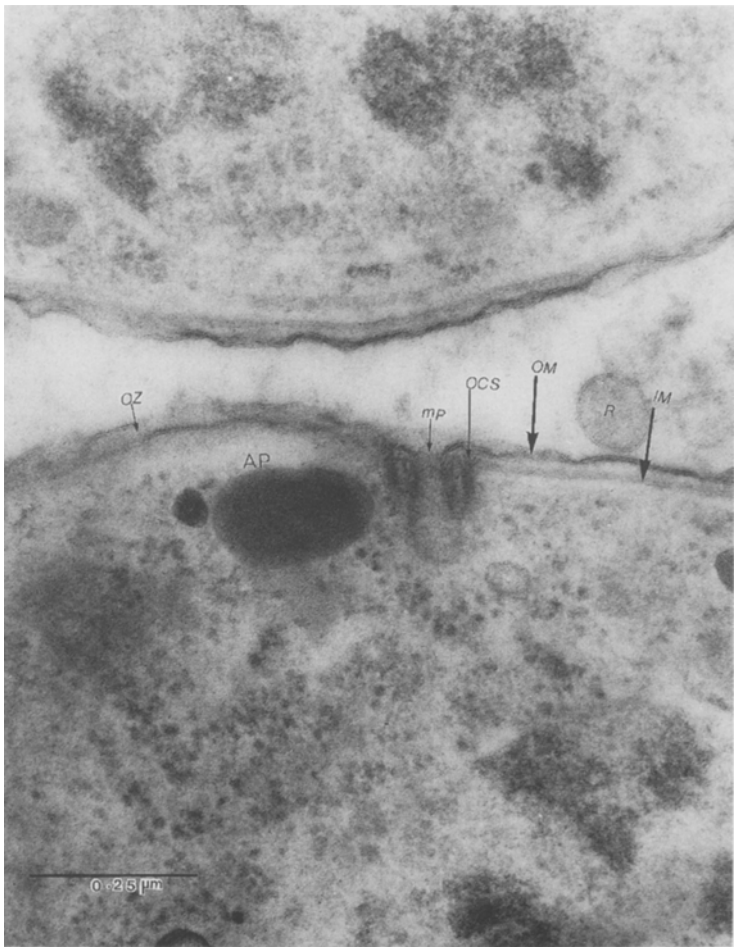

Figure 4. A micrograph of the fine details of a longitudinal section of the micropore (mp) of *Besnoitia besnoiti*. Note that the outer cylindrical structure (OCS) of the micropore appears to be principally formed from the inner membrane (IM) of the pellicle. The osmiophobic zone (OZ) separating the outer (OM) and inner membrane of the pellicle is uneven. A ribosome-like structure (R) was found free in the cyst fluid, while an amylopectin (AP) granule was observed in the cytoplasm. (×84 000)

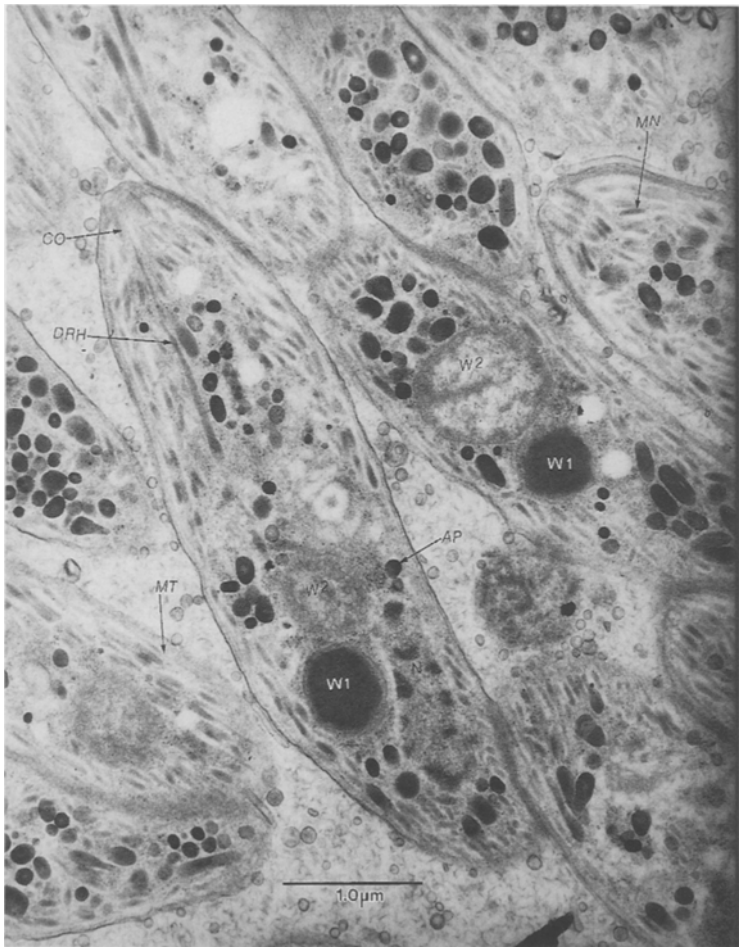

Figure 5. A micrograph of longitudinal and tangential sections of *Besnoitia caprae*. Note the membrane-bound wall forming body 1 (W1) on the left side and its closeness to the nucleus (N), and the start of formation of wall-forming body 2 (W2). The cystozoite on the right shows a W1 body which has shrunk and lost its limiting membrane and a well-formed W2 body. The conoid (CO), the micronemes (MN), the ducts of rhoptries (DRH), the microtubules (MT) and amylopectin (AP) granules can also be seen. (×17 500)

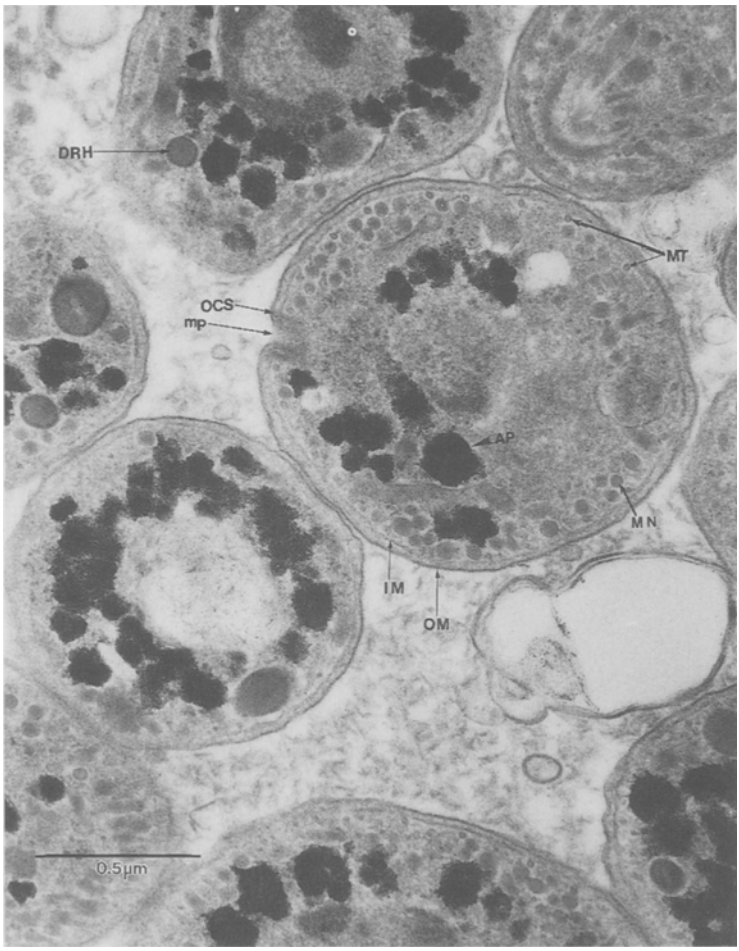

Figure 6. A micrograph of transverse and tangential sections of *Besnoitia caprae*. Note the smooth nature of the pellicle (P). The outer membrane (OM) and the inner membrane (IM) of the pellicle form the outer cylindrical structure (OCS) lining the micropore (mp). Also visible are the parallel, regularly arranged microtubules (MT), the ducts of rhoptries (DRH), the micronemes (MN) and a large number of amylopectin (AP) granules. (×28 000)

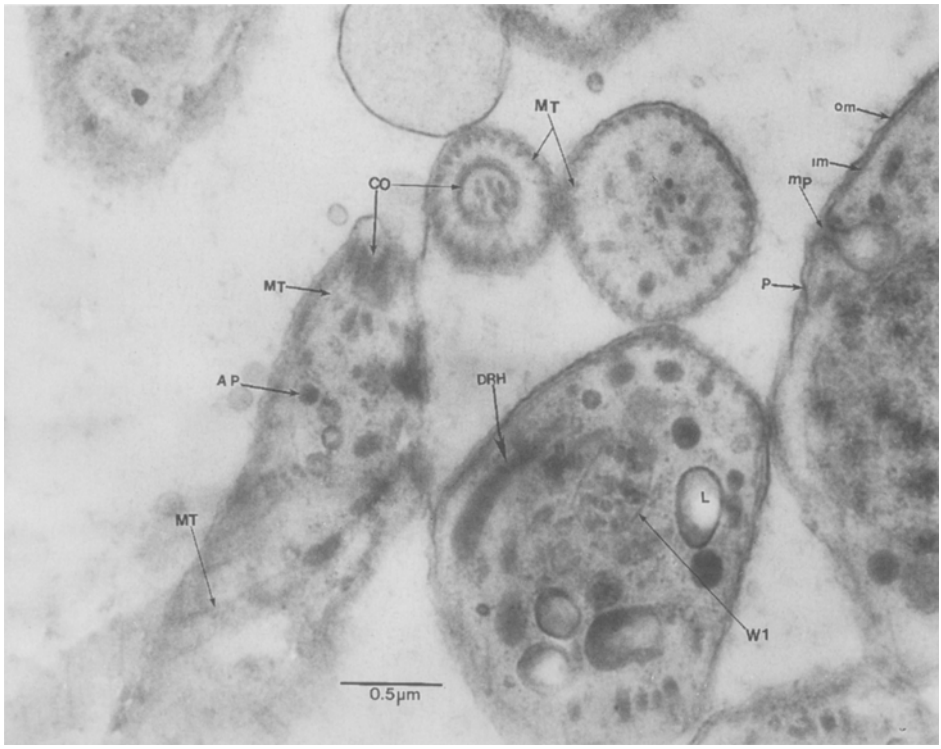

Figure 7. A micrograph of longitudinal, transverse and tangential sections of *Besnoitia besnoiti*. Note the conoid (CO) both in transverse and longitudinal sections, the wavy nature of the pellicle (P), the micropore (mp) formed by the outer membrane (om) and the inner membrane (im). The regularly arranged microtubules (MT) are straight at the anterior end but spiral posteriorly, as seen in the cystozoite on the left. A duct of rhoptry (DRH), an early wall-forming body 1 (W1), lipid (L) and amylopectin in granules (AP) are also present. (×35 000)

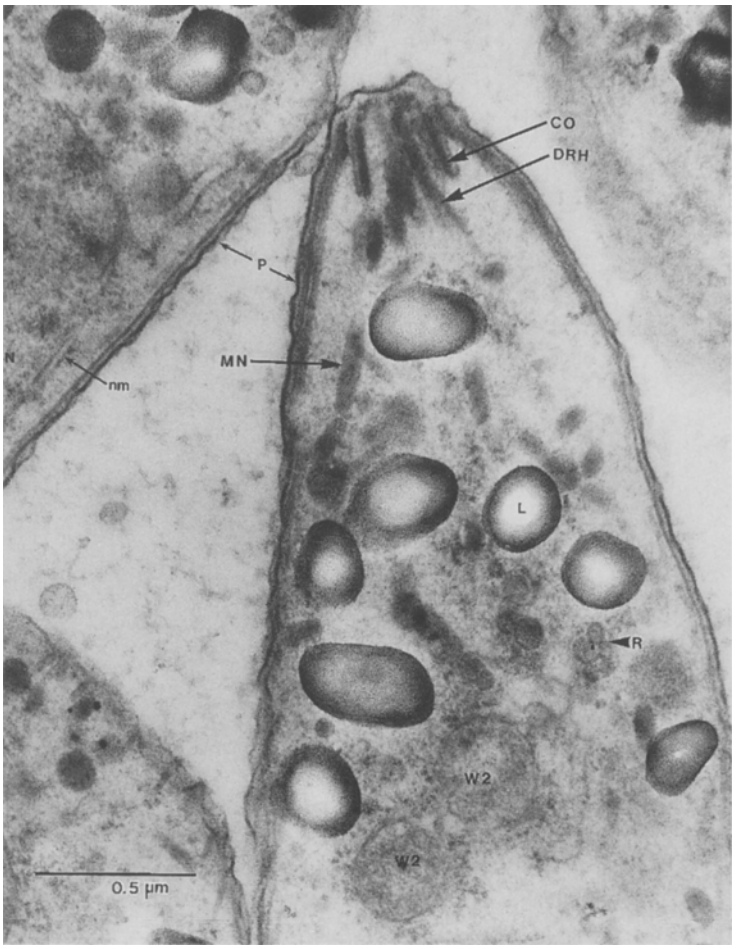

Figure 8. A micrograph showing a longitudinal section of a cystozoite of *Besnoitia besnoiti*. Note that the wall-forming body 2 (W2) seems to be dividing. Also visible are the ducts of rhoptries (DRH) originating inside the conoid (CO), the lipid granules (L), micronemes (MN), ribosome-like structures (R), the wavy pellicle and the nucleus (N) with nuclear membrane (nm). (×35 000)

There were 22 regularly arranged microtubules in both species that originated from the polar ring. These had an osmiophilic outer part and an osmiophobic centre. However, observation of longitudinal, transverse and tangenital sections of the cystozoites of the two organisms showed a marked difference in the direction of the microtubules. In *B. besnoiti*, the microtubules originate from the polar ring and run posteriorly, being straight for approximately the first one-sixth of the cystozoite, but then spiral before terminating on the posterior pole of the cystozoite. In *B. caprae*, the microtubules remain straight all the way from the polar ring to the posterior end of the cystozoite (Figures 1–4, 6 and 7).

Micronemes were found in large numbers in most parts of the cytoplasm. They were osmiophilic like the rhoptries, elongate–oval in shape and varied greatly in their lengths in both organisms.

Inside the cytoplasm of some of the cystozoites, one of two different kinds of wall-forming bodies were seen. The darker dense bodies were designated wall-forming body 1 (W1) and the spongy, honeycomb-like bodies were designated wall-forming body 2 (W2).

In *B. caprae*, the W1 bodies were round, membrane-bound, dark, dense and homogenous and were always found very close to the side of or anterior to the nucleus. The W1 bodies in *B. caprae* appeared to transform into W2 bodies and, as this happened, they lost the limiting membrane and shrank. The W2 bodies therefore started as loose spongy material without a well-defined boundary. They then became round or oval in shape, with a honeycomb-like internal appearance (Figure 5), and migrated anteriorly to rest half-way between the nucleus and the anterior end of the cystozoite. By this time, no traces of W1 were seen in the cystozoites. In two cystozoites, two W2 bodies were seen.

In *B. besnoiti*, the W1 bodies were membrane-bound, round and contained many smaller membrane-bound bodies. The closer the small bodies were packed, the darker the W1 body appeared. In *B. besnoiti*, W1 and W2 bodies were at times found in the same cystozoite on opposite sides of the nucleus. The W2 bodies in *B. besnoiti* were similar in appearance and shape to those of *B. caprae* and were also found halfway between the nucleus and the anterior end of the cystozoite (Figure 2). In one cystozoite, there seemed to be evidence that a W2 body was undergoing division (Figure 8).

Amylopectin and lipid droplets were found in the cytoplasm of cystozoites of both organisms. *B. caprae* appeared to have more amylopectin, represented by heavily osmiophilic dense homogenous bodies, while *B. besnoiti* appeared to have more lipids, represented by empty vacuoles, which were at times ringed with an osmiophilic zone.

The nucleus in both species was either oval or gourd-shaped and was found lying posteriorly below the centre of the cystozoite. In *B. caprae*, it was always surrounded by a halo zone. In *B. besnoiti*, the nuclear membrane was intact in most cystozoites. The chromatin in *B. caprae* appeared relatively homogenous, as opposed to that of *B. besnoiti*, which appeared granular (Figures 1 and 2).

Other organelles observed in the cytoplasm were mitochondria and ribosome-like structures. These were more readily demonstrable in *B. besnoiti* (Figure 2) than in *B. caprae*. The ribosome-like structures occurred either as single, double-membrane-bound structures, in pairs or as clusters surrounded by a membrane. In addition, ribosome-like structures were found being released from the anterior part of some cystozoites into the cyst fluid by exocytosis.

DISCUSSION

Both *B. besnoiti* and *B. caprae* have typical apicomplexan features, namely a three-layered pellicle, conoid, rhoptries, microtubules, micronemes and polar ring. Similar features were reported for the related organisms *Besnoiti jellisoni* (Sheffield, 1966, 1968) and *Toxoplasma gondii* (Ogina and Yoneda, 1966; Sheffield and Melton, 1968; Aikawa *et al.*, 1977). They were also previously reported for *B. besnoiti* (Neuman, 1974; Gobel *et al.*, 1985; Shkap *et al.*, 1988) and in a caprine *Besnoitia* in Kenya (Heydorn *et al.*, 1984).

The smooth pellicle in *B. caprae* compared to the wavy one in *B. besnoiti* explains the differences in width of the osmiophobic zone separating the outer and inner membranes of the pellicle in the two species.

The micropores of *B. besnoiti* and *B. caprae* were different in their structure. It therefore appeared that the two were different species, as micropore structural differences are specific (Scholtyseck and Mehlhorn, 1970).

The 22 regularly arranged microtubules (subpellicular fibrils) in the two species are of the same number found in *B. jellisoni* (Sheffield, 1966), in *T. gondii* (Sheffield and Melton, 1968) and in a caprine *Besnoitia* in Kenya (Heydorn *et al.*, 1984). The microtubules in *B. jellisoni* extended posteriorly beyond the region of the nucleus and, in tangential sections, appeared curved, indicating that they were spiralling around the cell periphery (Sheffield, 1966). *B. besnoiti* resembled *B. jellisoni* in the spiral arrangement of its microtubules. However, *B. caprae* differed from *B. jellisoni* because the microtubules in the former were straight.

The micronemes were highly pleomorphic and randomly arranged in both species, which indicates that they were highly convoluted. This agrees with previous reports on other sporozoans (Scholtyseck, 1970).

Wall-forming bodies are features of the macrogametes of all *Eimeria* species studied, in which two large granules, termed W1 and W2, fuse to form the typical two-layered oocyst wall of the microgamete. Granules of the first type, W1, are membrane-bound, homogenous and osmiophilic, and usually have a peripheral location. Those of the second type, W2, are spongy, fine structures mostly in the central region of the cytoplasm (Scholtyseck, 1970). In contrast, in *B. besnoiti* the W1 body is not homogenous but is composed of one large membrane-bound body with many small osmiophilic membrane-bound bodies. In *B. caprae*, the W1 body is similar to that described for *Eimeria* species. It was in all cases found in close proximity to the nucleus. In both the *Besnoitia* species, the W2 were similar to those reported for *Eimeria* species (Scholtyseck, 1970).

The relationship between the W1 and W2 bodies in *B. besnoiti* is not clear and needs further study. On the other hand, the W2 body in *B. caprae* appeared to be formed from the W1 body. The W2 body reported in this study resembles the unknown structure reported in *B. jellisoni* (Sheffield, 1966).

It is not clear why the W1 and W2 bodies have not been described in previous reports on *B. jellisoni* (Sheffield, 1966, 1968) and *B. besnoiti* (Neuman, 1974; Gobel *et al.*, 1985; Shkap *et al.*, 1988). However, most of these reports were based on cultured material, which may cause the parasite to behave differently. Also, where natural host cyst material was used (Shkap *et al.*, 1988), the cystozoites had been released from the cyst. During the present study, the W1 and W2 bodies were observed only in some cysts and within those cysts only in some cystozoites, so disruption could easily cause

one to miss the wall-forming bodies. The presence of two W2 bodies in cystozoites of *B. caprae* and a dividing W2 body in *B. besnoiti* may indicate that they have a role in replication. However, only serial ultrastructural studies can clearly elucidate the role and development of these bodies.

Whether the marked differences in the contents of amylopectin and lipids in the two species indicate that they have differences in their metabolism needs further investigation.

The nucleus of *B. caprae* was always surrounded by a halo zone; in the case of *B. besnoiti* it was in most cases, but not always, possible to discern the nuclear membrane. Processing of the tissues may have contributed to the apparent loss of the nuclear membrane. However, both sets of tissues were processed as a single batch and no trace of the nuclear membrane was seen in *B. caprae*. It is possible that this reflects the different lipid composition, but this question requires further research.

The cause of the granular appearance of chromatin in *B. besnoiti* and its relative homogeneity in *B. caprae* is not known. Further cytochemical, physiological, biochemical and serological studies would be needed to elucidate these and the other differing ultrastructural features observed.

It has been suggested that ultrastructural features should be used as the chief criteria for distinguishing sporozoans (Honigberg *et al.*, 1964; Garnham, 1969; Levine, 1969a,b). This ultrastructural study therefore supports the previously described difference in host range (Njenga *et al.*, 1993) and the continued use of the name *B. caprae*.

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