ULTRASTRUCTURE OF THE OOCYST WALL FORMATION IN *EIMERIA? BEAUCHAMPI* LEGER and DUBOSCQ, 1917 A COCCIDIAN PARASITE OF *GLOSSOBALANUS MINUTUS* (KOW.) (ENTEROPNEUSTA, HEMICHORDATA)

I. FERNANDEZ, J. BENITO, F. PARDOS

SUMMARY. The ultrastructure of the developing oocyst of *Eimeria? beauchampi* Leger and Duboscq, 1917 was studied from the ventral digestive epithelium of the hepatic region of *Glosso-balanus minutus* (Enteropneusta). A possible building mechanism of the oocyst wall is deduced and discussed from the present data and the available literature. During oocyst wall formation a total of 5 membranes were observed at or near the surface of the parasite among which some wall-forming materials will be stored. The origin and fate of such wall-forming materials are discussed and compared with data from other coccidians. The apparently full-formed wall is made up of an outer, dense layer, a median layer showing a labyrinthic-tubular lattice substruc-ture, and an inner, homogeneous and osmiophilic layer. A micropyle measuring 0.35 μm in dia-meter is also described.

Key-words: Ultrastructure. Oocyst wall. *Eimeria. Coccidia.*

Ultrastructure du développement de la paroi de l'oocyste d'*Eimeria? beauchampi* Leger et Duboscq, 1917, Coccidie parasite de *Glossobalanus minutus* (Enteropneusta, Hemichordata).

RÉSUMÉ. L'ultrastructure de l'oocyste d'*Eimeria? beauchampi* Leger et Duboscq, 1917 a été étudiée au cours de son développement dans l'épithélium digestive de la région hépatique chez *Glossobalanus minutus* (Entéropneuste). Le mécanisme de la formation de la paroi de l'oocyste est décrit. Au début du processus de formation, 5 membranes sont présentes autour de l'oocyste. Divers matériels se déposent ensuite dans la paroi, leur origine et leur emplacement final sont discutés et comparés avec d'autres Coccidies. Finalement la paroi de l'oocyste est constituée d'une fine couche externe osmiophile, d'une épaisse couche médiane à structure réticulée et d'une couche interne homogène et dense aux électrons. Un micropyle d'un diamètre de 0,35 μm est aussi décrit.

Mots-clés : Ultrastructure. Paroi de l'oocyste. *Eimeria. Coccidia.*

Introduction

Glossobalanus minutus (Kowalewsky) is an acorn worm occurring in intertidal sea bottoms. This and other species of enteropneusts have been reported to be natural hosts of sporozoan parasites, but literature on them is not abundant. So, Spengel (1893) superficially described some sporozoa in the digestive tube of *Glossobalanus sarniensis* and *Glandiceps talaboti*, and Leger and Duboscq (1917) found and named two species of *Eimeria, E? beauchampi* in the hepatic caeca and *E? epidermica* in the epidermis, both of them in *Glossobalanus minutus.*

Some light-microscopical studies have been carried out by us on this field: Benito (1977) studied the sexual forms of *Eimeria? beauchampi* with some histo-chemical information, and later, Fernandez and Benito (1983) published a paper on the several coccidian stages in the hepatic region of *G. minutus.* When using the electron microscope for the study of the hepatic region of this enteropneust, some developing oocysts were found in the ventral epithelium of this part of the digestive tract, together with two mature meronts which have been the object of a separate paper (in preparation).

As far as we know, this is the first time that the enteropneust parasites are studied electron-microscopically. We would like to remark the particular handicaps of working with this material whose characteristics prevent the use of current parasitologycal techniques, such as inoculation or incubation. That is why we depend on hazardous findings of single stages of the parasite life cycle, which is yet to be fully known.

Material and methods

The study material was obtained from adult specimens of the enteropneust *Glossobalanus minutus* (Kow.), collected from medium-coarse sand in the intertidal zone of Luanco (Asturias) and Noja (Santander) (North coast of Spain). Just captured they were dissected *in vivo* in order to obtain small tissue pieces which were immediately placed in fixative. Satisfactory fixation was obtained with: *a*) 4 % glutaraldehyde in sea water (6.5 g/l NaCl added) at 4º C, and *b*) 4 % gluta-raldehyde in Millonig phosphate buffer pH 7.4 made up with sea water (6.5g/l NaCl added). Both methods were followed by postfixation in 1 % osmium tetroxide in sea water (6.5 g/l NaCl added) and in Millonig phosphate buffer, respectively.

Following dehydration in graded acetone series, tissues were stained « in bloc » with uranyl acetate and embedded in Araldite via propylene oxide.

For light-microscopical observations, semi-thin sections were stained with toluidine blue. Thin sections were cut in a LKBIII ultramicrotome, stained with lead citrate (10 min) and photographed using a Philips EM201 electron microscope.

Results

Developing oocysts were found in hypertrophied host cells of the ventral epithelium of the gut in the hepatic region of *Glossobalanus minutus,* where three stages of the oocyst wall formation were observed. The parasites were found in close association with the host cell cytoplasm, without a patent vacuolar space. The host cell was a standard, non-secretory, epithelial cell type, which is widely distributed throughout the digestive tube. The irregularly shaped youngest stage was surrounded by a 0.155-0.238 μm thick primary cyst wall. The cytoplasm was found to be rich in free ribosomes and endoplasmic reticulum whose lamellae appeared associated with the electron-pale amylopectin granules constituting the main storage substance at this stage *(figs. 1, 2).* A small Golgi complex *(fig. 3)* could be observed in the vicinity of the large nucleus. Mitochondria with dense matrices and tubular cristae occurred mainly beneath the pellicle, and vacuoles of various thickness and contents were also present in the cytoplasm. On the other hand, a few dark bodies with a dense, granular substructure were seen in the periphery of the parasite *(fig. 4).* Smaller units, which could be derived from the particulation of such dark bodies appeared surrounded by endoplasmic reticulum membranes *(fig. 2)* and located immediately below the inner membrane of the pellicle.

At this developing stage the oocyst wall had 5 membranes (numbered 1 to 5 from outer to inner) *(fig. 2)*: membranes 1 and 2 occurred on the outer face of the wall layer; membranes 3 and 4 occurred on its inner face, and membrane 5 formed the limit of the parasite.

Exocytosis processes of the wall forming material were sometimes observed; the peripheral small units seem to release their contents into the space between membranes 2 and 3 to form the distinctly osmiophilic granular layer *(figs. 1, 4).*

In a more developed stage, oocysts were usually ovoid (25-27 μm long, and 14-16 μm wide) *(fig. 5)* and the wall (1 μm thick) was made up of three layers. The large nucleus which showed a high density contained a patent nucleolus, and in the more condensed cytoplasm numerous large lipid droplets and uniform dense granules were found. At this moment, the osmiophilic bodies which were seen in the earlier developing stage were absent.

Images showing deformation of the uniform dense granules in the parasite's periphery may suggest a breaking process into smaller units which assumed a position just below the pellicle *(figs. 6, 7).* Such small units fused with membranes 5 and 4 and released their contents into the space between membranes 3

Abbreviations used in the figures: A: amylopectin granules; AS: artificial space; DB: electron-dense bodies; DL: digestive lumen; ER: endoplasmic reticulum; GL: granular layer of the wall; HC: host cell; HN: host cell nucleus; IW: inner layer of the wall; L: lipid bodies; M1-M5: pellicular membranes; MW: median layer of the wall; N: nucleus; OD: outer layer discontinuities; OW: outer layer of the wall; SU: peripheral small units; UG: uniform dense granules; VS: vacuole-like spaces.
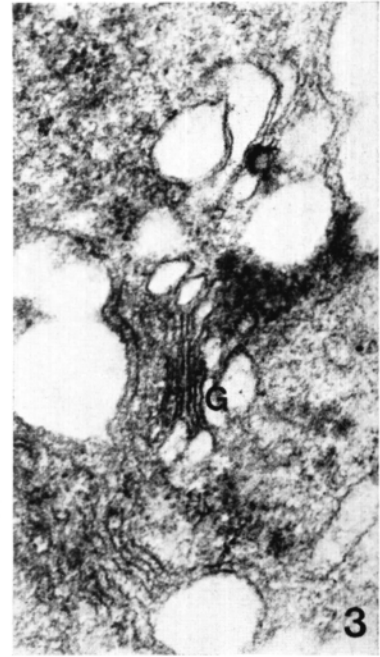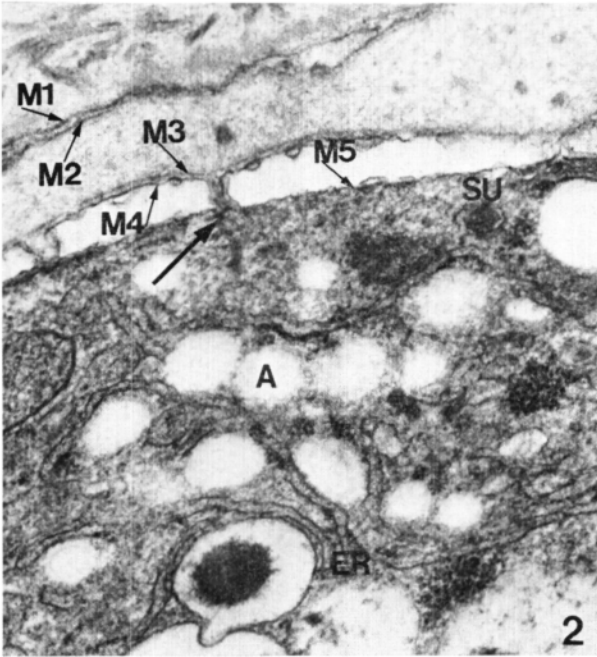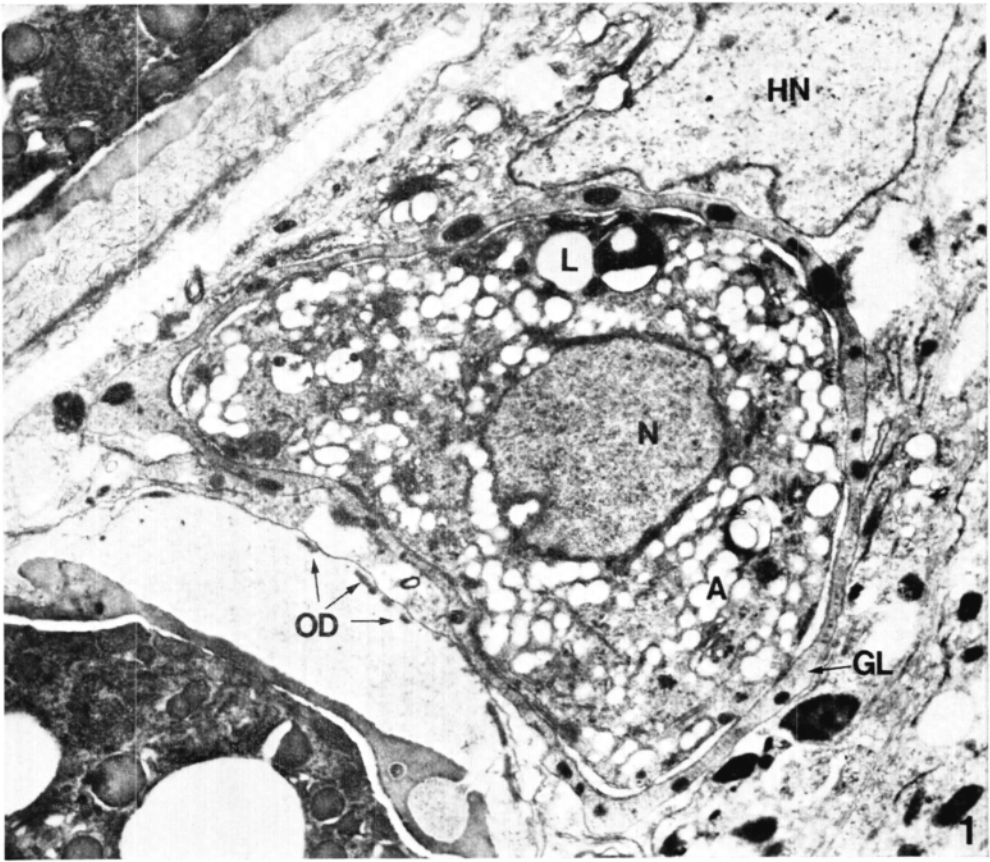
Fig. 1: Young oocyst in the digestive epithelium. Note the very close presence of a more developed stage in the same cell (below left) ($\times$ 10,600).

Fig. 2: Peripheral portion of a young oocyst. Extrusion of dense material can be seen (arrow) ($\times$ 37,400).
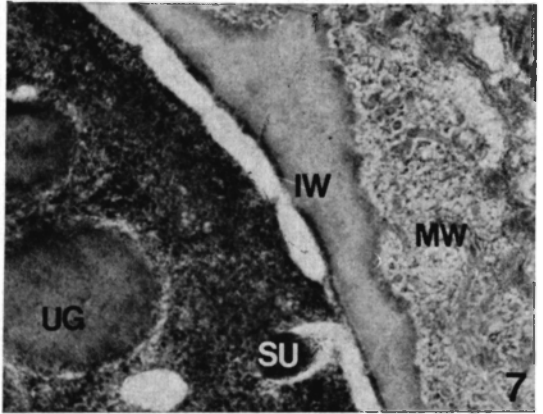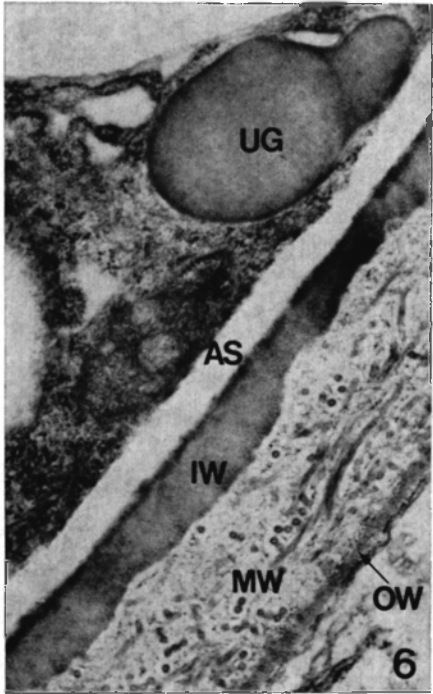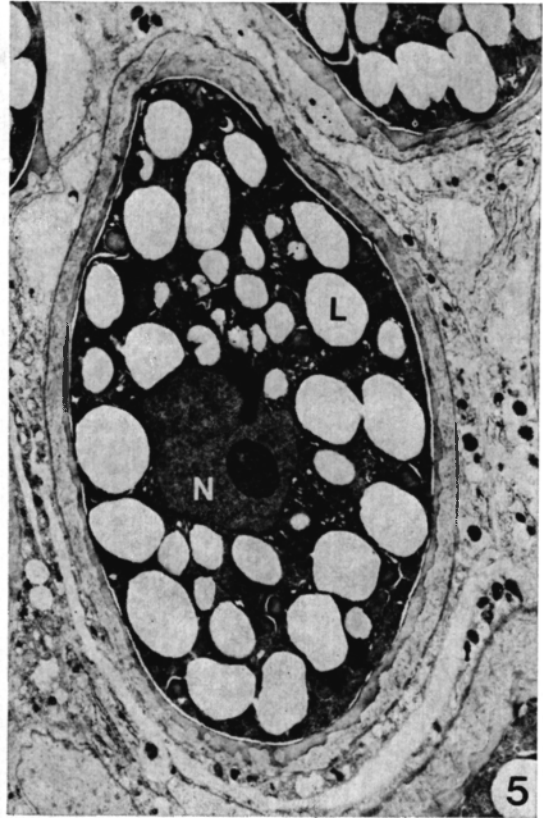
Fig. 3: Detail of the Golgi apparatus ($\times$ 57,000).

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Fig. 4: Partial view of the young oocyst ($\times$ 12,800).
Fig. 5: A more developed stage of the oocyst formation ($\times$ 4,000).
Fig. 6: Detail of the parasite periphery showing a presumably particulating dense granule ($\times$ 37,400).
Fig. 7: Partial view of the periphery of the parasite showed in figure 5. A small unit is located beneath the plasmalemma ($\times$ 37,000).

MW

IW

SU

8

OW

MW

IW

9

MW

IW

VS

10

IW

OW

MW

11

12

DL

HC

HC

13
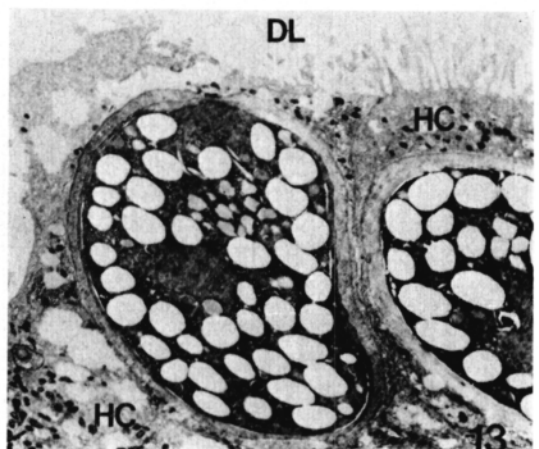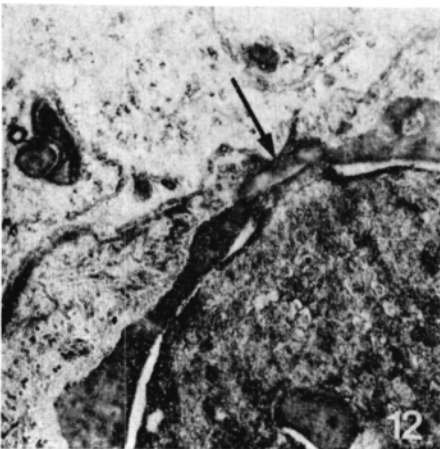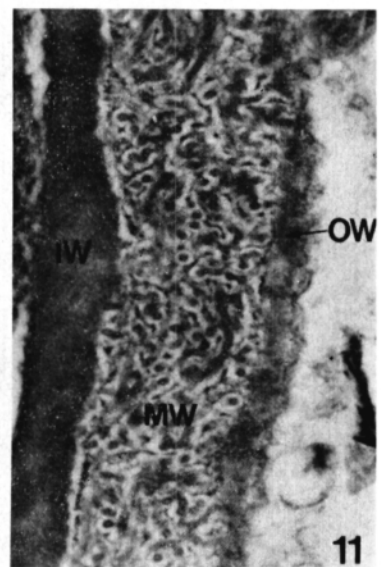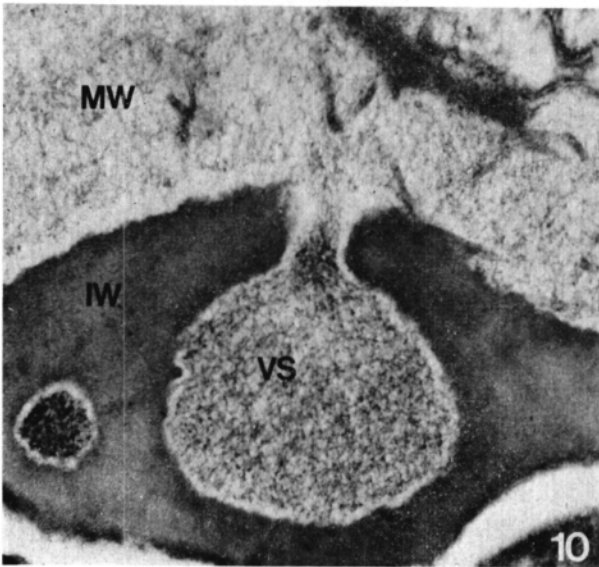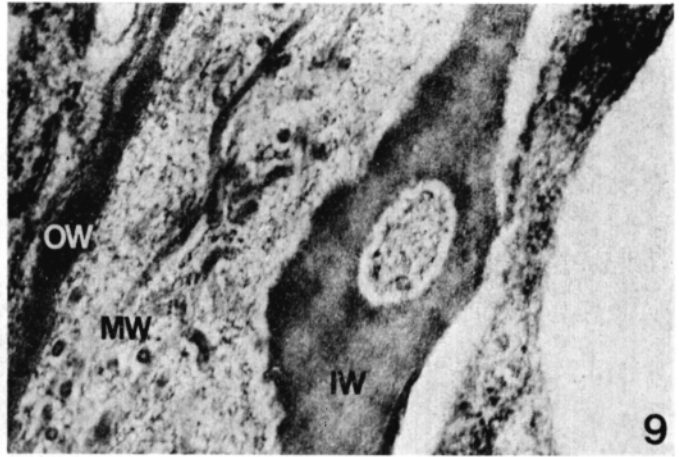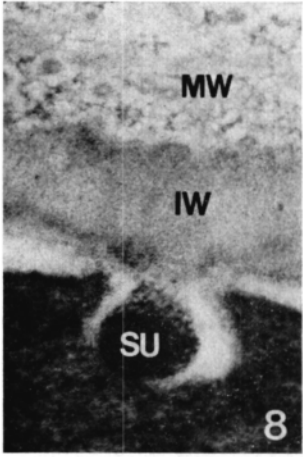
and 2 of the oocyst wall *(figs. 7, 8)*. The outer layer of the wall, immediately located under membranes 1 and 2, presumably aroses from the fusion of the osmiophilic material of the earlier granular layer *(figs. 1, 4)*. At this stage the outer layer showed some discontinuities, the median layer was made up of a tubular material randomly arranged and embedded in a filamentous matrix *(figs. 6, 9, 10)* and the inner layer had an irregular surface and was built up of a uniform, electron-dense material resembling the outer layer. On the other hand, vacuole-like spaces containing filamentous materials were found inside the inner layer. Extrusion of this material into the median layer could also been detected *(figs. 1, 10)*.

The aparently full-formed wall of the parasite showed the following structure: firstly, an outer, relatively thin, dense layer (OW), 0.116 μm thick, just beneath membranes 1-2; secondly, a median tubular layer (MW), 0.464 μm thick, and thirdly, an inner layer (IW), 0.208 μm thick, formed by a homogeneous osmiophilic material. Now, the outer layer is a continuous sheet; the median layer is made up of a strongly packed labyrinthic-tubular lattice, and the inner layer shows a uniform thickness. The outer and the median layers become thinner gradually towards an area identified as a micropyle (0.55 μm diameter), whereas the inner layer seems to form a cap-like structure over the micropyle *(fig. 12)*.

When the wall became fully formed, the oocysts appeared to be rejected into the digestive lumen *(fig. 13)*.

Discussion

The occurrence of *Eimeria? beauchampi* in the digestive epithelium of *Glossobalanus minutus* has been already reported by Leger and Duboscq (1917), Benito (1977), Fernandez and Benito (1983). The features showed by the present parasites lead us to believe that we are dealing with developing oocyst stages.

Although the oocyst wall formation in *Eimeria? beauchampi* mainly supports the observations from other species of *Eimeria*, the structure of the oocyst wall differs from other studies. Changes in the wall-forming bodies found in most of *Eimeriina* macrogametes, as well as a membrane proliferation from the parasite surface, are repeatedly shown to be involved in the wall formation process. The wall forming bodies have been originally reported as belonging to two different

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Fig. 8: Higher magnification of a small unit releasing its contents into the wall (× 48,600).
Fig. 9: Parasite wall showing the median layer with tubular structures embedded in a fibrillar matrix (× 50,100).
Fig. 10: Vacuole-like space with fibrillar material inside releasing its contents into the median layer (× 45,200).
Fig. 11: Final structure of the median layer. Note the strongly packed labyrinthic-tubular lattice (× 43,100).
Fig. 12: Detail of the micropyle (arrow). Note the decreasing thickness of the wall towards it (× 16,740).
Fig. 13: Mature oocyst being rejected into the digestive lumen (× 2,750).

types, WF1 and WF2 (Scholtyseck and Voig, 1964; Scholtyseck *et al.*, 1969). It is generally assumed that wall formation occurs by disaggregation and/or fusion of the wall forming bodies and later transference of the resulting material to the surface where they are stored between the membranes which enveloped the zygote (Scholtyseck, 1973; Scholtyseck *et al.*, 1969; Scholtyseck *et al.*, 1971; Varghese, 1975; Ferguson *et al.*, 1975; Ferguson *et al.*, 1977; Michael, 1978; Chobotar *et al.*, 1980; Elwasila, 1982). Dense granules resembling the wall-forming bodies of *Eimeria* species have been observed in several species of *Isospora* (Ferguson, 1980), *Sarcocystis* (Entzeroth *et al.*, 1985) and *Goussia* (Paperna *et al.*, 1986). However, several coccidian species have been reported to show no wall-forming bodies (Davies, 1978; Paterson and Desser, 1981; McLean, 1984).

The deposition process of the wall forming material seems to be very similar in most coccidians. It has already been pointed out that wall forming bodies I are responsible for the formation of the outer layer of the oocyst wall, whereas the wall forming bodies II built up the inner layer. In the present earliest developing stages, the peripheral units which seem to arise from the fragmentation of dense bodies (presumably WF1) release their contents into the wall and, according to Scholtyseck (1973), the extrusion occurs by an exocytosis mechanism.

The data from numerous studies of wall-formation show that following the changes in the WF1, such development and the formation of the pellicular membranes of parasites are closely related. In this study, 5 membranes have been observed in the formation process of the oocyst wall. Because of the absence of a patent vacuolar space around the parasite, it is difficult to say if the membrane 1 of the present material actually corresponds to the membrane of the parasitophorous vacuole. On the other hand, it seems probable that membrane 5 is the plasmalemma of the parasite. The exocyted material is deposited between membranes 2 and 3. In these young developing stages, the presence of dense bodies in the cytoplasm is poor, and both they and the peripheral small units are going to dissapear in further stages.

Five membranes were also described by Sibert and Speer (1980) in the oocyst wall of *Eimeria nieschulzi* and by Chobotar *et al.*, 1980 in *E. papillata*. However, the number and origin of the developing membranes vary in other eimerian species (Dubremetz and Yvore, 1971; Lee and Millard, 1971; Doens-Juteau and Senaud, 1974; Wheat *et al.*, 1975; Ferguson *et al.*, 1977; Michael, 1978; Senaud *et al.*, 1980; Elwasila, 1981; Gajadhar *et al.*, 1986).

The numerous electron-dense granules which occur in the cytoplasm of more developed stages of *Eimeria? beauchampi* have a homogeneous structure as the WFII bodies of *E. magna* (Speer *et al.*, 1973) and *E. falciformis* (Knöber *et al.*, 1980). The presumed WFII material of *E? beauchampi* appears to particulate within small vesicles which release their contents between membranes 3 and 2. However, the origin and differentiation process of the two layers, both the median and the inner which occur between such membranes, could not be observed, although vacuole-like spaces containing a filamentous material inside like the one found in the median layer, have been detected in the inner layer. Furthermore, some

images observed suggest the releasing of the vacuolar material into the median layer.

The apparently fully developed wall of the parasites here studied is composed of three layers, although most of the eimerian studies reveals that the oocyst wall is made up of two layers. However, this number seems to be highly variable among coccidian. On the other hand, the thickness of the oocyst wall has been related to the environmental conditions (Lom, 1971).

The first ultrastructural observation of a coccidian micropyle has been reported by Sibert and Speer (1980) in mature oocysts of *Eimeria nieschulzi*. Later, Gajadhar *et al.* (1986) pointed out the presence of a micropyle in *E. truncata*. In the present study, the micropyle was closed by a homogeneous material resembling the one forming the inner layer of the wall.

The importance of the oocyst characters for systematic purposes at the level of coccidian genera has been pointed out by Long and Joiner (1984). These authors argued that the shape of the oocyst is a further specific character, whereas the size has no systematic value because of its high variability. This view was stated earlier by Marquardt (1981) who remarks the caution which is necessary when using the oocyst structure for systematic purposes.

From its morphological features and having in mind the parasite specificity (Marquardt, 1981; Long and Joyner, 1984), we have identified the parasite studied here as *Eimeria? beauchampi* Leger and Duboscq, 1917. Both sexual and asexual stages have been already described by us (Benito, 1977; Fernandez and Benito, 1983) light-microscopically. Unfortunately, when using the electron microscope only developing oocysts here described and mature meronts have been found.

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