FINE STRUCTURE OF THE DEVELOPMENT OF *SARCOCYSTIS SINGAPORENSIS* IN *PYTHON RETICULATUS* FROM MACROGAMONT TO SPORULATED OOCYST STAGE

PAPERNA I.* & MARTELLI P.**

Summary :

Three, 4-month old reticulated pythons (*Python reticulatus*) hatched from eggs laid by a newly caught female from Singapore Island, were fed on muscles of *Sarcocystis singaporensis*-infected *Rattus rattus* caught in Singapore. Snakes were sacrificed five, six and eight days later. The infected tissues were studied by transmission electron microscope. The present communication summarizes findings on macrogamont and oocyst stages. In the premature stages, rough endoplasmic reticulum consolidate into a large rectangular array; the electron-dense wall-forming-like bodies reveal a laminar structure. Macrogamont parasitophorous vacuoles became filled with granular matrix and electron-dense strands, which later on consolidate into a coat around the fertilized zygote. The oocyst wall is constructed from several formed membranes combined with deposited substance. All development to the sporulated oocyst stage occurs in the mucosal epithelium.

KEY WORDS : *Sarcocystis singaporensis, Python reticulatus,* macrogamonts, oocysts, ultrastructure.

Résumé : ÉTUDE ULTRASTRUCTURALE, CHEZ LE PYTHON RÉTICULÉ, DU DÉVELOPPEMENT DEPUIS LE MACROGAMONTE JUSQU'À L'OOCYSTE SPORULÉ, DE *SARCOCYSTIS SINGAPORENSIS*

*Trois pythons réticulés (*Python reticulatus*), âgés de quatre mois, éclos à partir d'œufs pondus par une femelle, peu après sa capture, à Singapour, ont ingéré des muscles de* Rattu rattus *capturés à Singapour et infectés par* Sarcocystis singaporensis. *Les Serpents furent sacrifiés cinq, six et huit jours plus tard. Les tissus infectés ont été étudiés au microscope électronique. Cette note résume les observations sur le macrogamonte et sur les différents stades de l'oocyste. Dans les stades immatures, le reticulum endoplasmique granuleux occupe une large zone rectangulaire. Les corps précurseurs de la paroi sont denses aux électrons et présentent une structure laminaire. Les vacuoles parasitophores des macrogamontes se remplissent d'une matrice granuleuse formant des trainées denses aux électrons. Elle se condense ultérieurement en une enveloppe autour du zygote. La paroi de l'oocyste est formée de plusieurs membranes et de la substance déposée. L'ensemble du développement s'effectue dans l'épithélium de la muqueuse.*

MOTS CLÉS : Sarcocystis singaporensis, Python reticulatus, *macrogamontes, oocystes, ultrastructure.*

INTRODUCTION

Zaman & Colley (1975) presented electron microscopic images of mature microgamonts, macrogamonts and young oocysts of *Sarcocystis singaporensis,* from killed, naturally infected free ranging reticulated pythons (*Python reticulatus*) from Singapore. The only other detailed, fine-structural account on the endogenous development of *Sarcocystis* in snakes is that of *S. muriviperae* described from both *Vipera palaestinae* and *Coluber jugularis* (Paperna & Finkelman, 1996). All other fine structural accounts on endogenous stages from reptile hosts are restricted to the late-stage oocysts (Abdel-Ghaffer *et al.*, 1990; Mehlhorn & Matuschka, 1986; Matuschka *et al.*, 1987). In this communication, we present an ultrastructural study of macrogamont development into sporulated oocysts from timed experimental infections of reticulated pythons.

MATERIALS AND METHODS

Young, four-month-old, 60-70 cm long, 80-90 g reticulated pythons, reared from eggs laid by a newly caught female from Singapore Island, were force-fed with infected muscles removed from *Sarcocystis singaporensis*-naturally infected free-ranging *Rattus rattus* trapped in the forested area around Singapore Zoological Gardens (Paperna & Martelli, in press). Infection with *S. singaporensis* was confirmed by histological examination of the muscle sarcocysts. Snakes were sacrificed five, six and eight days after feeding, with chloroform. The gut was removed and sections along the digestive tract were examined by light microscope by squashing a small fresh piece of gut

* Department of Animal Sciences, Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot 76100, Israel.
** Veterinary Hospital, Singapore Zoological Gardens, 80 Mandai Lake Rd., Singapore.
Correspondence: I. Paperna.
Tel: 972-8-9489945 – Fax: 972-8-9465763.
E-mail: paperna@agri.huji.ac.il

tissue between slides. Small pieces from loci in the gut found to host infection were fixed for electron microscopy.

For transmission electron microscopy (TEM), gut segments were fixed in 2.5 % glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 24 h at 4°C, rinsed repeatedly in the same buffer, post-fixed in 1.0 % osmium tetroxide in the same buffer for 1 h and, after rinsing in the buffer, dehydrated in graded ethyl alcohols and embedded in Agar 100 medium (Agar Scientific, Ltd., U.K.). Thin sections, cut on a Reichert Ultracut microtome with a diamond knife, were stained on grids with uranyl acetate and lead citrate, and examined with a Jeol 100CX TEM.

RESULTS

Macrogamonts, zygotes and and oocysts developed within the epithelial layer lining the anterior gut and the small intestine.

Young macrogamonts, observed from day 5 post feeding, were lodged in a double-walled parasitophorous vacuole (PV). The inner lamina of the PV often folded into protrusions which sloughed off into the PV lumen (Fig. 1). The macrogamonts were bound by a fine, bilayered membranous wall and were readily recognizable by their homogeneous nucleus, which was sometimes elongated, and a conspicuous nucleolus (Figs. 1, 2). The cytoplasm contained mitochondria, endoplasmic reticulum (ER) and at the perinuclear zone, a large, whorled Golgi-like (paragolgi) organelle (Figs. 2, 3). Growing macrogamonts developed variable-size wall-forming-like electron-dense bodies (Figs. 3, 4). When these bodies became very large, they revealed special laminar structure (Figs. 5, 6). Small electron-dense bodies seen within the perimeter of the Golgi-like organelles (Fig. 3) might be the anlagen of the wall-forming-like bodies.

In growing macrogamonts, rough ER formed a dense network (Figs. 3, 4) which gradually consolidated into a large array of parallel tubules, situated alongside the nucleus (Figs. 6, 7).

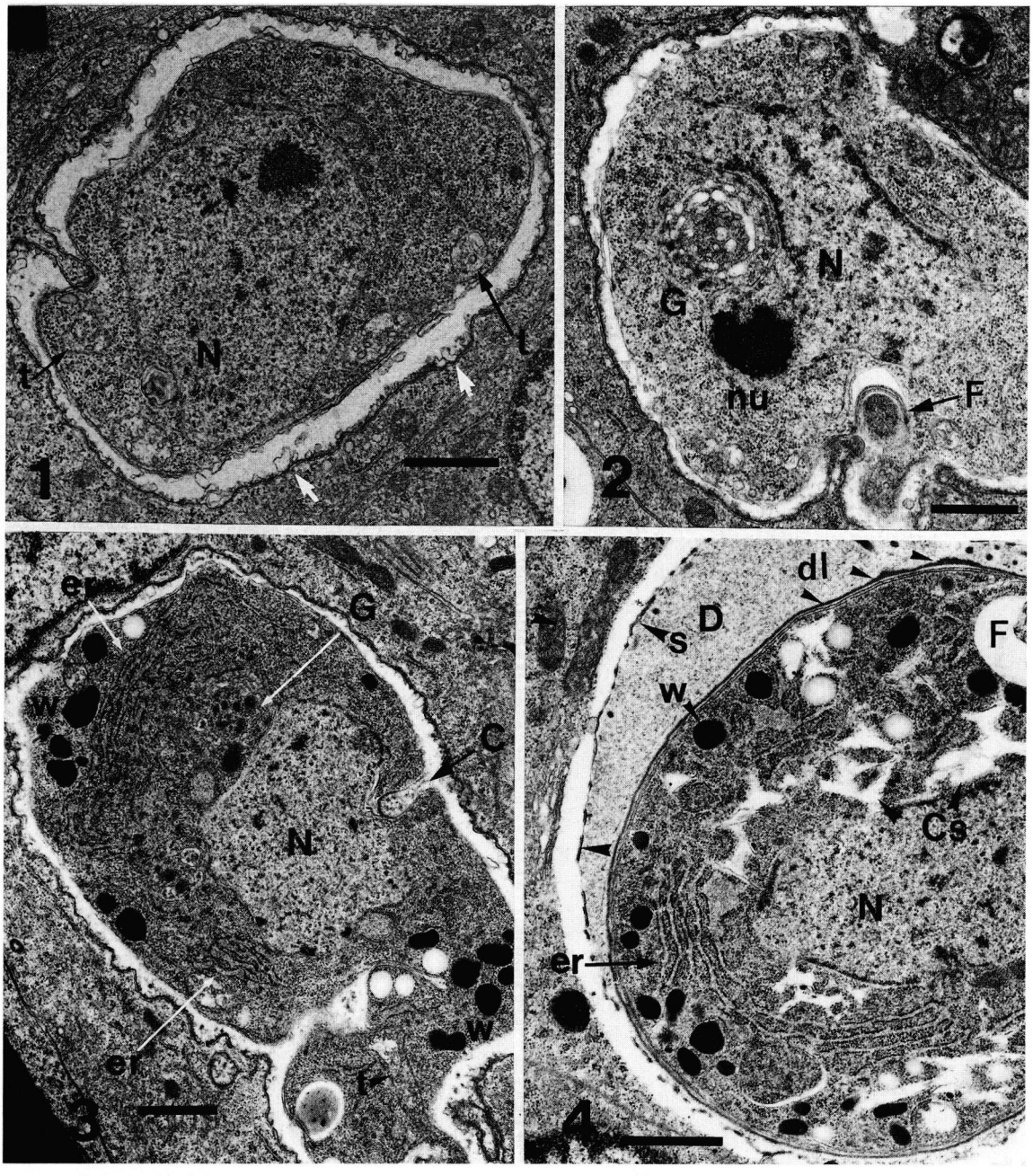
In both young and growing macrogamonts it was possible to locate a cytostome or feeding invagination, sometimes in connected to food vacuole (Figs. 2, 3). Some of the larger vacuoles contained laminated or crystal-like objects (Figs. 4, 6). The large spaces formed in some macrogamonts apparently due to processing faults contained outlines of canaliculi (Fig. 4).

The bilaminate PV wall which usually seen folded or slouthed remained intact in some later-stage images (Fig. 5). As the macrogamont grew, its PV lumen filled up with a medium-density matrix. Electron-dense strands occurred along the PV boundary (Fig. 7) and within the matrix, filling its lumen (Figs. 4, 8). Up to this stage, the macrogamont retained a bilaminate membranous boundary which incorporated micropyles (Fig. 8). This wall became overlaid with a granular electron-dense coat (Fig. 8).

Amylopectin granules increased in number only after the formation of zygotes (Figs. 9, 10), which became predominant in the snake sacrificed on day 6 post-feeding. The cytoplasm also contained canaliculi (Fig. 10), an adnuclear body (Fig. 11), and retained a variable number of wall-forming-like bodies (Fig. 9). The electron-dense layer coating the zygote, was gradually displaced (Fig. 10) in the young oocyst by a series of formed membranes (Figs. 9, 10); the two outermost membranes (M1, 2), possibly incorporating some of the material from the former electron-dense coat (Figs. 11, 12), appeared to form the future outer oocyst wall. A space formed between the two underlying membranes (M3 and 4) (Fig. 10) was gradually filled with fine globules (Figs. 13, 14) apparently to be eventually consolidated as the oocyst's inner wall. As the oocyst differentiated, first the PV, and subsequently the entire host cell disintegrated: the PV borders dissolve, leaving the PV matrix in scattered inclusions within the host cell cytoplasm (Figs. 9, 11). With the host cell's degradation and the displacement pressure of the growing oocyst, the adjacent host cell became compressed (Figs. 12, 13). All development to this stage occurred in the host's mucosal epithelium, as evident from the presence of desmosomes (Figs. 12, 13).

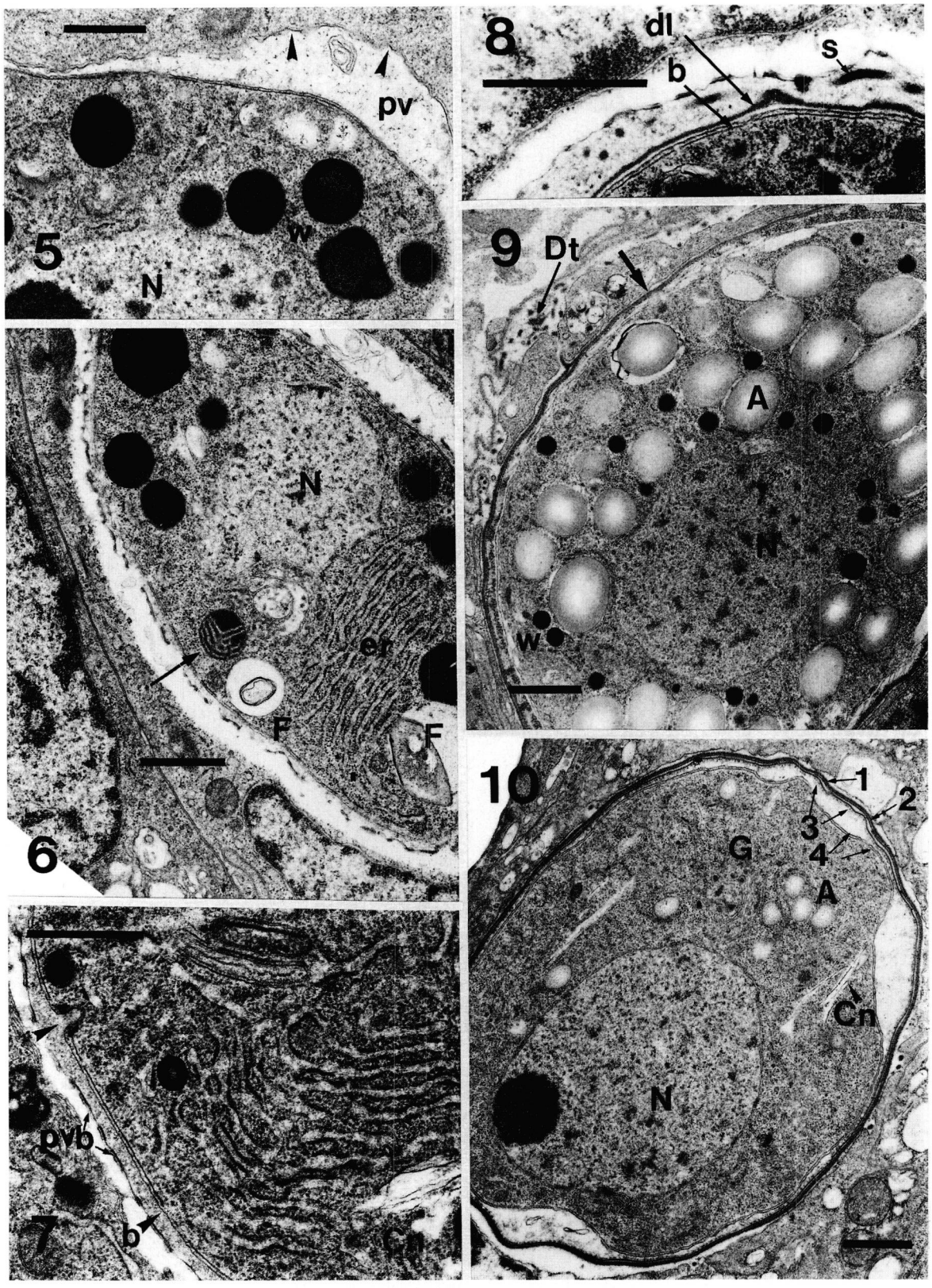
Zygotes with remains of microgametes embedded between their two outermost unit membranes occurred in the snakes sacrificed six and eight days post infection (alongside non-sporulated and sporulated oocysts). They were seemingly defunct or of arrested development (Figs. 15, 16). They were coated by an outer, heavy, electron-dense layer; one was still located within the intact, matrix-filled PV (Fig. 15), the other's PV was already in disarray (Fig. 16). Fully formed oocysts were enclosed in a thick, seemingly hard (as evidenced by shrinkage after processing), outer wall of medium electron density, overlaying a thin, electron-dense inner wall (Fig. 17). The oocyst body, loaded with amylopectin granules was bordered by several membranes which were indistinguishable from one another.

Observed sporulated oocysts contained two nondifferentiated sporocysts. The oocyst seemed to be located extracellularly alongside a large inclusion of PV filling substance (Fig. 18). The outer face of the oocyst wall was hardened, while its innner layers appeared to separate into membranal strands impregnated with granular substance (Fig. 19). In the lumen formed around the sporocyst, granular matrix and crystalloid particles accumulated (Figs. 20., 21). The sporocyst wall

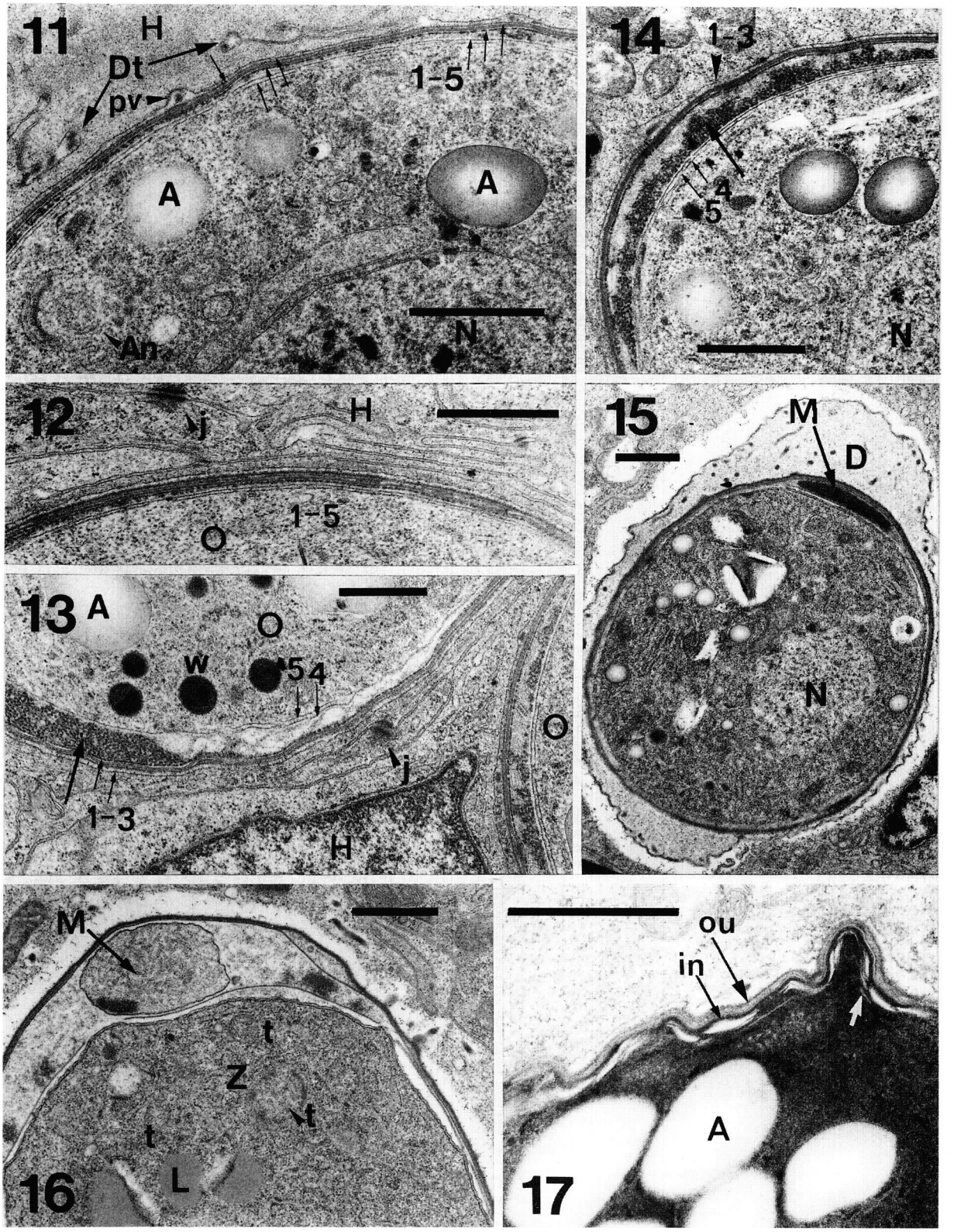


Figs. 1-4. – Macrogamonts of *Sarcocystis singaporensis* in gut epithelial cells.
1. Young macrogamont, protrusions of the PV wall sloughing off into the lumen (white arrows). 2. Young macrogamont with elongate nucleus, Golgi-like organelle and food vacuole. 3. Growing macrogamont, already with formed wall-forming-like bodies. 4. Mature macrogamont, already with cisternal or canalicular spaces, PV is filled with granular matrix with electron dense deposits along the PV and the macrogamont walls.

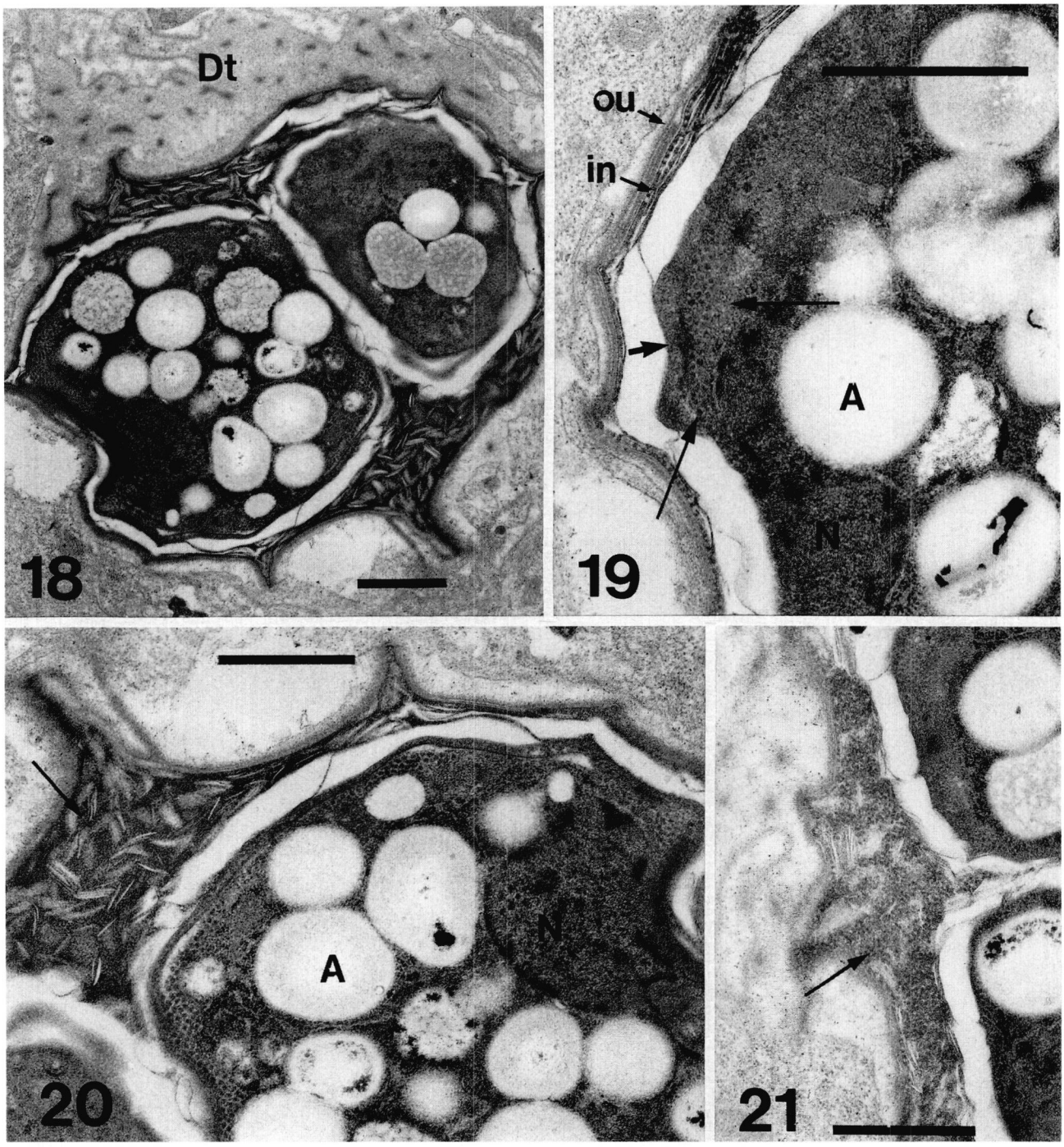
Abbreviations. – 1, 2, 3, 4, 5, oocyst wall membranes; **A**, amylopectin granules; **An**, adnuclear organelle; **b**, macrogamont's border membranes; **C**, cytostome; **Cn**, canaliculi; **Cs**, cisternae or canaliculi; **D**, PV filling granular matrix; **dl**, electron-dense coat; **Dt**, inclusion of granular matrix with electron-dense strands; **er**, endoplasmic reticulum; **F**, food vacuole; **G**, Golgi-like organelle; **H**, host cells; **in**, oocyst inner wall; **j**, desmosome; **L**, lipid vacuoles; **M**, microgamete residue; **N**, nucleus; **nu**, nucleolus; **O**, oocyst; **ou**, oocyst outer wall; **pv**, parasitophorous vacuole; **pvb**, granular matrix; **s**, electron dense strands; **sw**, sporocyst wall; **t**, mitochondria; **w**, wall-forming-like bodies; **Z**, zygote (scale = 1 µm).



Figs. 5-10. – Mature macrogamonts and zygotes of *S. singaporensis.*
5. Macrogamont with developed wall-forming-like bodies in PV lined with fine bilayered wall (arrows). 6. Mature macrogamonts with ER array; note laminated wall-forming-like body (arrow). 7. Enlarged view of the macrogamont ER array; also showing details of the PV border, and the granular matrix, the macrogamont's border and the micropyle (arrow). 8. View of the zygote wall's electron-dense coat, the PV granular matrix and the electron-dense strands. 9. Zygote, arrow: the forming wall and the electron dense coat. 10. Zygote already enclosed by four membranes.



Figs. 11-17. Development from zygote to oocyst.
11. Zygote's wall membrane (1-5) appear, while the PV matrix scatters into the host cell cytoplasm. 12. The walled young oocyst grows against the compressed adjoining epithelial cells showing conspicuous desmosomes. 13,14. Further stage in wall formation: wall material accumulates between membranes 3 and 4 (arrow). Note the still present wall-forming-like bodies. 15. Oocyst with microgamete residue, enclosed in matrix-filled PV. 16. Oocyst with microgamete residue – detailed view of the enclosing wall membranes; the PV has disintegrated, and its matrix dispersed. 17. Oocyst encased in a bilayered (ou and in) hard wall, arrow: inner plasmalemmal bounding membranes.



Figs 18-21: Sporulated oocysts.
18. Oocyst with two sporocysts, located alongside an inclusion of PV filling substance. 19. Detail of the oocyst wall: the wall hard on the outside and its innner layer separate into membranal strands impregnated with granular substance; the sporocyst is bound by a hard wall (thick arrow); note the densely packed granules in the cytoplasm (long arrow). 20. Crystalline deposits (arrow) between the walls. 21. Granular deposite (arrow) between the oocyst and sporocyst walls.

was homogeneously thick. In the dense sporocyst cytoplasm, it was possible to trace nuclei, ER, amylopectin granules and plaques of densely packed granules.

DISCUSSION

Gamogonous development of only two species of *Sarcocystis* from snakes has been studied to date (Zaman & Colley, 1975; Paperna & Finkelman, 1996). These are comparable to species developing in mammalian definitive hosts (see Scholtyseck & Hillali, 1978; Becker *et al.*, 1979; Mehlhorn & Heydorn, 1979; Entzeroth *et al.*, 1985), and to one of unknown host from tissue culture (developed from sarcocysts in grackles, *Quiscalus quiscula* by Vetterling *et al.*, 1973). The described macrogamonts shared a few common features, but at the same time demonstrated a good number of unique ones, either species-specific or possibly also derived from being at various stages of differentiation. Upon fixing, premature macrogamonts from snakes assume variable, sometimes deeply invaginated shapes, suggesting them to be more mobile in their PV *in vivo* than those of mammalian hosts and of grackle origin, which after fixation, assume a round to oval shape.

A large array of rough ER arranged parallel was the most conspicuous fine structural feature in the presently described premature macrogamonts; the ER does not aggregate to such an extent in any of the other studied *Sarcocystis* spp. In *S. muriviperae*, expansion of the ER tubules (seen as numerous cisternae, Paperna & Finkelman, 1996) could have obliterated the arrayed configuration of the reticulum.

Golgi-like organelles – comprised of small array of tubular whorls occur both in young macrogamonts and late stage zygotes (as adnuclear organelle). Images of *S. suihomonis* zygotes contain two types of wall-forming-bodies (Mehlhorn & Heidorn, 1979); as they occur in eimeriid coccidia; in other *Sarcocystis* sp. including *S. singaporensis* only a single type of wall-forming-like body present, also referred to as "dense granules" or "bodies" (Vetterling *et al.*, 1973; Entzeroth *et al.*, 1985). Viewing the fine structural evidences available to date, the species-specificity of their configuration is certain. Less certain (see Mehlhorn & Heydorn, 1979; Entzeroth *et al.*, 1985) is their claimed role in wall formation (Vetterling *et al.*, 1973; Zaman & Colly, 1975). A seemingly anlagen of wall-forming-like bodies within the perimeter of a Golgi-like organelle recalls the suggested role of the Golgi system in the formation of type 1 wall-forming bodies in eimerians (Scholtyseck *et al.*, 1971). In *Sarcocystis*, the wall-forming-like bodies disappear, either together or only after oocyst wall consolidation. In fish coccidia, where the oocyst wall is usually as soft as in *Sarcocystis* spp., either one or two types of wall-forming-like bodies also occur, and their implication in wall formation remains, likewise, uncertain (Paperna, 1995).

The PVs of all described *Sarcocystis* are filled with a fine granular substance with typical electron dense-strands. There is some interspecific variation in the matrix density and strand particle texture; and in the timing of their formation and disaggregation. It persists in some species PVs to the stage of oocyst maturity (Mehlhorn & Heydorn, 1979; Entzeroth *et al.*, 1985; Paperna & Finkelman, 1996) and is saved in separate extravacuolar inclusions even beyond sporulation (present study). The electron dense-strands seem to contribute the substance for the electron-dense layer which coats the mature macrogamont. This layer may act in preventing excessive impregnation of the zygote: it becomes very conspicuous around zygotes penetrated by microgametes and disappears in early oocysts. Material from this layer also appears to become incorporated into the consolidating outer oocyst wall (Mehlhorn & Heydorn, 1979; present study).

In the presently studied species, as in most previous descriptions, zygote images contained microgametocyte remains between their wall membranes, evidence of a fertilization process unique to *Sarcocystis* (Sheffield & Fayer, 1980).

The process of membrane assembly into oocyst wall formation assumes a pattern similar to that observed in the previously described species (see Mehlhorn & Heydorn, 1979; Entzeroth *et al.*, 1985; Paperna & Finkelman, 1996).

Zaman & Colley (1975) and Brehm & Frank (1980) report finding of *S. singaporensis* oocysts in the lamina propria. In histological and semithin material from eight day old experimental infection and also of natural infections (Paperna & Martelli, unpublished) zygotes as well as sporulated oocysts were seen located within the mucosal epithelium above the basal membrane. In the TEM images the borders of the host cells surrounding the forming oocysts contained desmosomes. Sporulated oocysts were seen to be located within the epithelial layer also in a histological image presented by Brehm & Frank (1980; Fig. 6c) from day eight post-infection.

The displacement of zygotes and young oocysts from the epithelial layer to the lamina propria is regarded as characteristic of all *Sarcocystis* spp. (Entzeroth *et al.*, 1985). However, the process of displacement in *Sarcocystis* remains unexplained. Dissolution of the host cell lodging the ripening oocysts, seen in this study, suggests that zygotes vacate their host-cell and migrate independently (see Entzeroth *et al.* 1985). The sporulated oocyst located in the epithelial appeared to be

already extracellular, It was located alongside an inclusion containing residues of the substance once filling the PV. This substance apparently fulfills some function in the oocyst eviction process. Similar large "pools" of a same substance has been seen around evicted oocysts of *S. muriviperae* (Paperna & Finkelman, 1996). The material accumulating within the cavity formed between the oocyst and the sporocyst wall appears to be the by-product of oocyst wall disintegration.

REFERENCES

ABDEL-GHAFFER F. RAHMAN, BASHTAR A., BADR ASHOUR M. & SARKAN Th. Life cycle of *Sarcocystis gongyli* Trici 1911 in the skink *Chalcides ocellatus ocellatus* and the snake *Spalerosophis diadema. Parasitological Research,* 1990, *76,* 444-450.

BECKER B., MEHLHORN H. & HEYDORN A.O. Light and electron microscopic studies on gamogony and sporogony of five Sarcocystis species *in vivo* and in tissue cultures. *Zentralblatt fuer Bakteriologie Mikrobiologie und Hygiene, I. Abt Orig.*, 1979, *A244,* 394-404.

BREHM H. & FRANK W. Der Entwicklungskeislauf von *Sarcocystis singaporensis* Zaman and Colley, 1976 im End- und Zwischenwirt. *Zeitschrift fuer Parasitenkunde*, 1980, *62,* 15-30.

ENTZEROTH R., CHOBOTAR B. & SCHOLTYSECK E. Electron microscopic study of gamogony of *Sarcocystis muris* (Protozoa, Apicomplexa) in the small intestine of cats. *Protistologica,* 1985, *21,* 399-408.

MATUSCHKA F.R., HEYDORN A.O., MEHLHORN H., ABD-AL-AAL Z., DIESING L. & BICHLER A. Experimental transmission of *Sarcocystis muriviperae* n.sp. to laboratory mice by sporocysts from the Palestinian viper (*Vipera palaestinae*): a light and electron microscope study. *Parasitology Research,* 1987, *73,* 33-40.

MEHLHORN H. & HEYDORN A.O. Electron microscopical study on gamogony of *Sarcocystis suihominis* in human tissue culture. *Zeitschrift fuer Parasitenkunde*, 1979, *58,* 97-113.

MEHLHORN H. & MATUSCHKA F.R. Ultrastructural studies of the development of *Sarcocystis clethrionomyelaphis* within its final and intermediate hosts. *Protistologica,* 1986, *22,* 97-104.

PAPERNA I. Ultrastructure and developmental affinities of piscine coccidia. *Diseases of Aquatic Organisms*, 1995, *22,* 67-76.

PAPERNA I. & FINKELMAN S. Ultrastructural study of *Sarcocystis muriviperae* development in the intestine of its snake hosts. *Folia Parasitologica,* 1996, *43,* 13-19.

PAPERNA I. & MARTELLI P. *Sarcocystis* in rats and pythons on Singapore Island. *In:* Cowan D., Feare C. (eds.) Advance in Vertebrate Pest Management. Proc. first European Vertebrate Pest Management Conference, 1999 (in press).

SCHOLTYSECK E., MEHLHORN H. & HAMMOND D.M. Fine structure of macrogamonts and oocysts of coccidia and related organisms. *Zeitschrift fuer Parasitenkunde*, 1971, *37,* 1-43.

SCHOLTYSECK E. & HILLALI M. Ultrastructural study of the sexual stages of *Sarcocystis fusiformis* (Railliet, 1897) in domestic cats. *Zeitschrift fuer Parasitenkunde,* 1978, *56,* 205-209.

SHEFFIELD H.G. & FAYER R. Fertilization in the coccidia: fusion of *Sarcocystis bovicanis* gametes. *Proceedings of the Helminthological Society of Washington,* 1980, *47,* 118-121.

VETTERLING J.M., PACHEDO N.D. & FAYER R. Fine structure of gametogony and oocyst formation in *Sarcocystis* sp. in cell culture. *Journal of Protozoology,* 1973, *20,* 613-621.

Zaman V. & Colley F.C. Light and electron microscopic observations of the life cycle of *Sarcocystis orientalis* sp. n. in the rat (*Rattus norvegicus*) and the Malaysian reticulated python (*Python reticulatus*). *Zeitschrift fuer Parasitenkunde,* 1975, *47,*169-185.

Reçu le 18 mars 2000
Accepté le 15 juin 2000