

The functional cytology of *Bonamia* sp. (Haplosporidia) infecting oysters *Tiostrea chilensis*: an ultracytochemical study

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ABSTRACT: *Bonamia* sp., a serious pathogen of oysters *Tiostrea chilensis*, was studied using the zinc iodide-osmium tetroxide technique (ZIO), the imidazole-osmium technique for lipids (IM), and reactivity for acid phosphatases [β -glycerophosphatase (β -GPase), cytidine monophosphatase at pH 5.0 (CMPase 5.0) and pH 2.7 (CMPase 2.7), thiamine pyrophosphatase (TPPase)], and β -galactosidase to try and determine the function of cytoplasmic organelles. ZIO reacted with the nuclear membrane, Golgi, endoplasmic reticulum (ER), cytoplasmic vesicles, and variably with putative phagosomes, the mitochondrial matrix, haplosporosomes and multivesicular bodies, but not lipid bodies. IM strongly labelled lipid bodies, and lipids in the nuclear membrane, ER except anastomosing ER (aER) and mitochondrial membranes and cristae, but was weak in aER and on haplosporosomes. β -GPase occurred in the nuclear membrane, some elements of ER, Golgi and mitochondria, and in lipid bodies. CMPase 5.0 was variable in Golgi and ER, but was stronger at pH 2.7 and occurred in the nuclear membrane. TPPase only occurred in a single Golgi cisterna, or very weakly in a few lipid bodies. β -galactosidase was rarely seen, and only within Golgi. As there was considerable metabolism of unsaturated lipids and little hydrolytic enzyme content, it was concluded that *Bonamia* sp. may synthesise lipids from triglycerides hydrolysed by haemocyte enzymes, reducing the need for parasite hydrolytic enzymes.

KEY WORDS: *Bonamia* sp. · Organelle function · Hydrolytic enzymes · Lipid metabolism

INTRODUCTION

Elucidation of the fine structure of an organism is of limited value if the functions of its constituent parts are unknown. Protists have some organelles similar to those of higher eukaryotes, and ontogenetic, although not necessarily functional, homology can be assumed. Other organelles are restricted to protists and their functions are unknown. Such an organelle is the haplosporosome of haplosporidians, which appears to be formed from interaction of putative nuclear material with Golgi-like arrays and endoplasmic reticulum (ER) (Perkins 1979, Hine 1992, Hine & Wesney 1992). The spherulosome of haplosporidian spores may also form haplosporosomes, and be homologous to the Golgi apparatus (Desportes & Nashed 1983). Haplosporosomes contain periodate-reactive complex carbohydrates (Perkins 1976), and have a protein matrix, an external membrane of glycoprotein, and an internal

membrane of lipid (Azevedo & Corral 1985). Other organelles, called lipid or dense bodies, are formed from concentric arrays of smooth endoplasmic reticulum (Hine 1992), and could contain acid phosphatase (Hervio et al. 1991, Hine 1992).

This study was to determine whether *Bonamia* sp. has a Golgi-endoplasmic reticulum-lysosome system similar to that of higher eukaryote cells (Kornfeld & Mellman 1989), and to examine the functions of this system. Golgi-endoplasmic reticulum relationships were studied using the zinc iodide-osmium tetroxide (ZIO) technique, and lipid distribution using an imidazole-buffered osmium technique (Benchimol & de Souza 1985). β -glycerophosphatase, cytidine monophosphatase (CMPase) and β -galactosidase were used as lysosomal enzyme markers. CMPase was selected because it stains *trans*-Golgi networks on the *trans* aspect of Golgi in higher animals (Herme et al. 1991), and CMPase and β -galactosidase as they have been

reported from *B. ostreae* (Hervio et al. 1991, Hervio 1992). Thiamine pyrophosphatase was used because it selectively stains Golgi *trans* cisternae in higher animals (Herme et al. 1991).

MATERIALS AND METHODS

From December 1992 to August 1993, the hearts of 226 oysters *Tiostrea chilensis* infected with *Bonamia* sp., as determined from heart imprints (Hine & Wesney 1992), were fixed for the following techniques.

Zinc iodide-osmium tetroxide (ZIO) for subcellular structures (Pellegrino de Iraldi 1977). Fixation: 2.5% glutaraldehyde in 0.22 μ m filtered seawater (FSW) for 1 h at 18°C. Wash: FSW. Process as described by Benchimol & de Souza (1985). Incubate control grids for 30 min at 18°C with 1 mM dithiothreitol (DTT) dissolved in 0.1M Tris buffer (pH 6.8) before ZIO incubation (Benchimol & de Souza 1985).

Imidazole-buffered osmium tetroxide (IM) for lipids (Angermüller & Fahimi 1982). Fixation: 2.5% glutaraldehyde in FSW. Wash: 2 \times FSW. Post-fixation: 2% OsO₄ in 0.1 M imidazole (IM) buffer, pH 7.5, for 30 to 60 min at 18°C (Benchimol & de Souza 1985).

Techniques used for enzyme cytochemistry were as described below, with 2 routine modifications: (1) 0.22 μ m FSW was used as buffer for glutaraldehyde fixation, and for the 2 subsequent washes and the second wash after incubation, while buffers specified in published techniques were used for incubation and the first post-incubation wash; and (2) grids were post-fixed in 1% OsO₄ for 20 min at 18°C to minimise osmophilia.

β -Glycerophosphatase (β -GPase) (Robinson & Karnovsky 1983). Fixation: 1 h at 4°C in 2% glutaraldehyde. Wash: 2 \times FSW. Incubation: 30 min at 37°C in Gomori medium [1 mM β -glycerophosphate, 2 mM cerium chloride, 5% sucrose, 0.1 M Na acetate-HCl buffer (pH 5.0)], change incubation solution with fresh solution and incubate for further 30 min, first post-incubation wash with 0.1 M Na cacodylate buffer with 5% sucrose.

Cytidine monophosphatase (CMPase) (Hervio et al. 1991). Fixation: 1 h at 4°C in 2% glutaraldehyde.

Wash: 2 \times FSW. Incubation: 90 min at 37°C in Novikoff's medium (25 mg cytidine 5'-monophosphate, 12 ml DH₂O, 3 ml 1% lead nitrate, 10 ml Na acetate buffer, 20% sucrose), first post-incubation wash with 0.05 M Na acetate buffer. Incubation and first post-incubation washes were carried out at pH 2.7 (CMPase 2.7) or the usual pH 5.0 (CMPase 5.0).

Thiamine pyrophosphatase (TPPase) (Robinson & Karnovsky 1983). Fixation: 1 h at 4°C in 2% glutaraldehyde. Wash: 2 \times FSW. Incubation: 1 h at 37°C in 1 mM thiamine pyrophosphate chloride, 2 mM manganese chloride, 2 mM cerium chloride, 0.1 M Tris-maleate buffer (pH 7.2) to make 100 ml and 20% sucrose; first post-incubation rinse in Tris-maleate buffer.

β -Galactosidase (Rhodes & Blom 1986). Fixation: 30 min at 4°C in 1.25% glutaraldehyde. Wash 2 \times FSW. Incubation: 5 h at 37°C in a medium of 8 mg 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 2 ml dimethyl formamide, 32 mg spermidine tri-hydrochloride, 2 ml 0.085% NaCl, 12 ml 0.05 M potassium (hexa) ferrocyanide, 124 ml 0.1 M Na acetate buffer (pH 5.4). First post-incubation wash in Na acetate buffer.

Controls. For β -GPase, CMPase, and TPPase, grids were incubated without substrate, or with 0.05 mM Na L-tartrate as inhibitor (+ta). Beta-galactosidase was incubated without substrate. Oyster haemocytes served as positive controls. Grids were either examined unstained, or after 5 min staining with 5% lead citrate (Pb cit).

Organelle nomenclature is that used earlier (Hine 1991a, 1992, Hine & Wesney 1992).

RESULTS

Reaction patterns in *Bonamia* sp. organelles are given in Table 1. The ZIO technique stained the nuclear membrane (Figs. 1 & 2), but not nuclear pores (Fig. 3), and stained perinuclear Golgi (Fig. 2), short, meandering (Fig. 1) and large parallel arrays (Figs. 4 & 5) of smooth endoplasmic reticulum (sER) including sER around haplosporosome-like bodies (Fig. 4). Lipoid bodies (Fig. 5), anastomosing endo-

Figs. 1 to 8. *Bonamia* sp. Zinc iodide-osmium tetroxide (ZIO) labelling. Fig. 1. A late stage *Bonamia* sp. with a ZIO-positive nuclear membrane, peripheral endoplasmic reticulum, ER, anastomising ER, aER (arrow), and ER (*) around a lipoid body (L). Pb cit, \times 16 610. Fig. 2. Portion of parasite showing the nuclear membrane (NM), Golgi (G), aER, ER and lipoid body (LB). Pb cit, \times 41 625. Fig. 3. Nuclear membrane showing unreactive nuclear pores (arrowheads). Unstained, \times 44 000. Fig. 4. Portion of late stage *Bonamia* sp. showing stacks resembling smooth ER, sER, vesicles (V), haplosporosome-like bodies (HLB), and bizarre membraneous configurations with a ZIO-negative dense lamina between confronting cisternae (arrowhead). Pb cit, \times 45 130. Fig. 5. Golgi-like cisternae (G) associated with a lipoid body (LB). Unstained, \times 55 390. Fig. 6. aER closely associated with a peripheral reticulated structure (RS). Pb cit, \times 72 120. Fig. 7. Putative endocytotic vacuole (E). Note the ruffled parasite surface adjacent to the vacuole, and variability of haplosporosome (H) staining. Pb cit, \times 52 140. Fig. 8. Multivesicular bodies near the parasite surface. Pb cit, \times 47 080

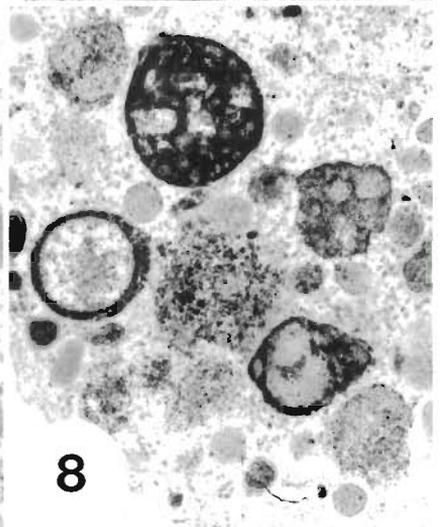
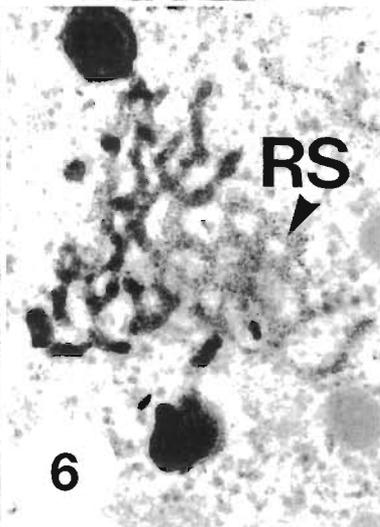
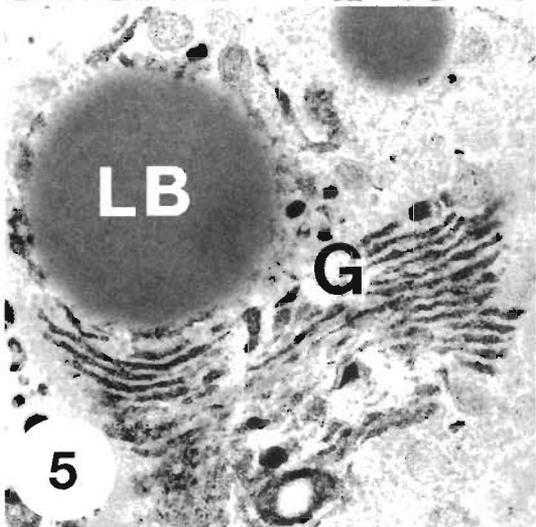
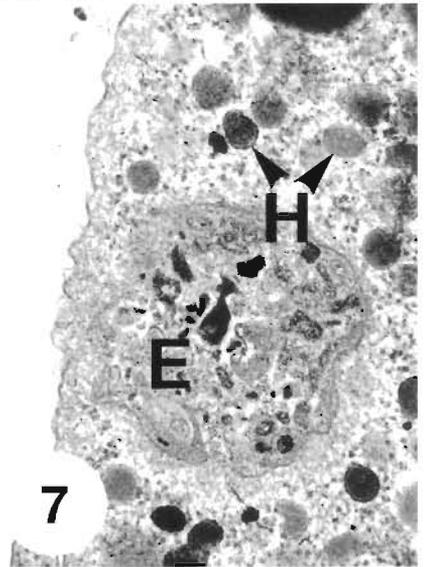
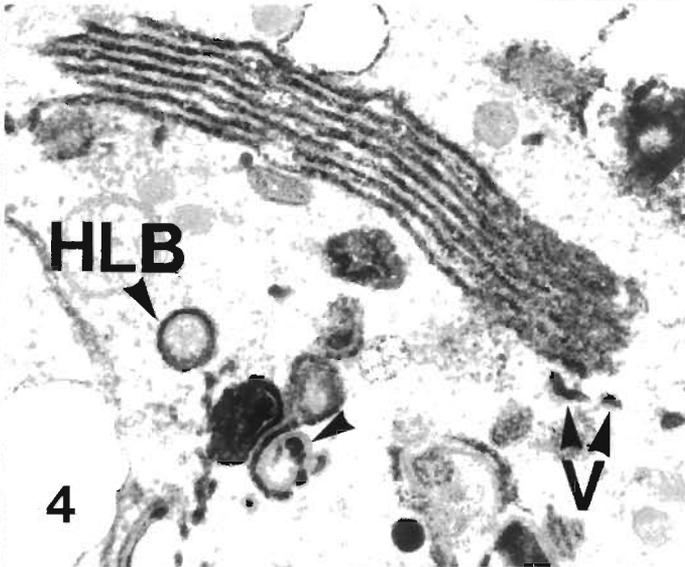
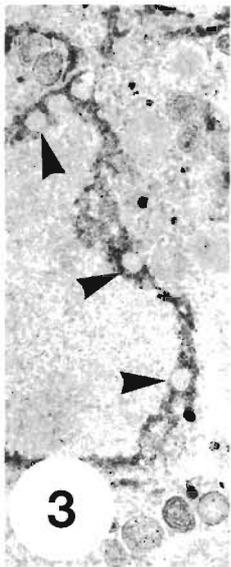
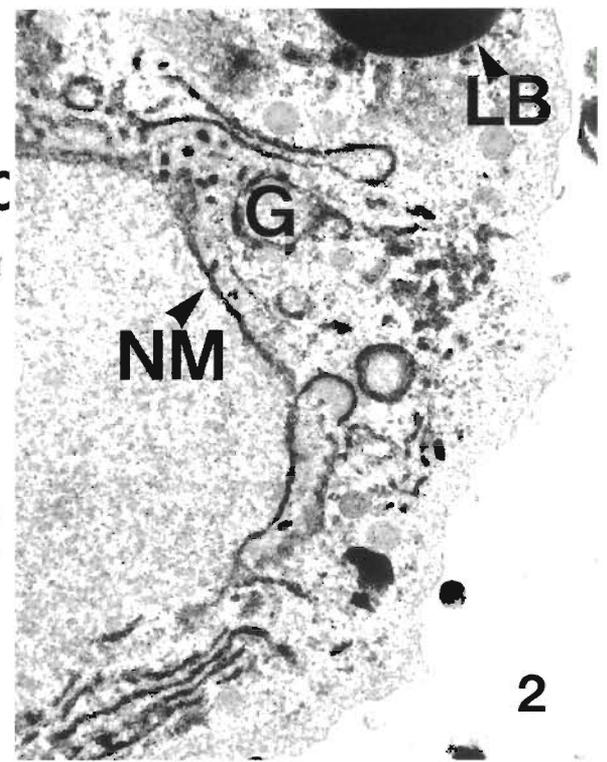
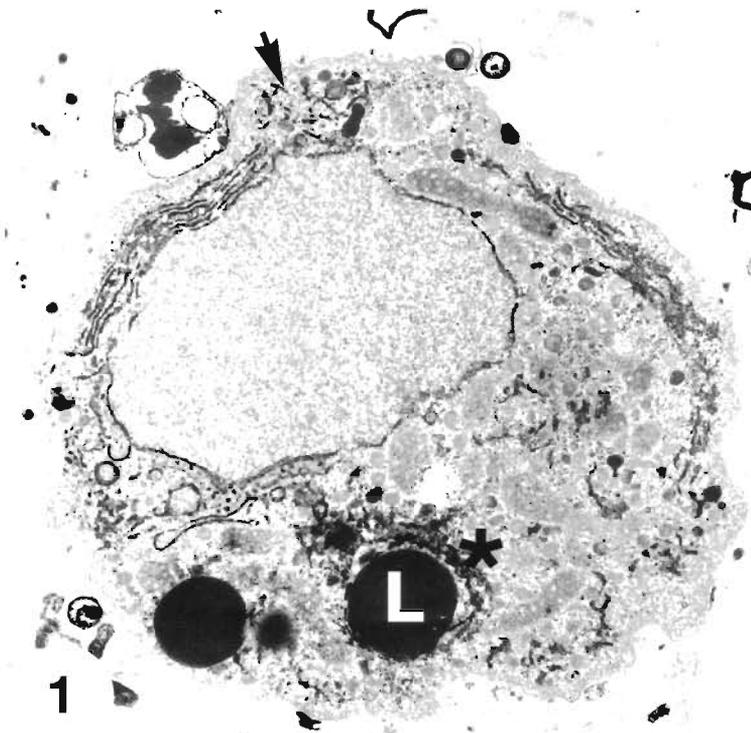


Table 1. *Bonamia* sp. Cytochemical reactions in organelles. ++: moderate to strong; +: weak; ±: variable; -: not observed; ta: L-tartrate added as inhibitor; ns: peripheral Golgi not seen; nd: not determined. For cytochemical abbreviations see 'Materials and methods'

	ZIO	IM	β-GPase	CMPase 5.0	CMPase 2.7	TPPase	β-Galactosidase
Nuclear membrane	++	++	++ta	-	++	-	-
Perinuclear Golgi	++	±	±ta	±	++	±	±
Peripheral Golgi	++	++	ns	ns	ns	ns	ns
Endoplasmic reticulum (ER)	++	++	++ta	±	++	-	-
Anastomosing ER	++	+	-	±	++	-	-
Lipoid bodies	±	++	+	nd	nd	-	-
Haplosporosomes	±	+	-	-	-	-	-
Mitochondrial membranes	-	++	++ta	-	-	-	-

plasmic reticulum (aER) which appeared to connect to a reticular structure (Fig. 6), cytoplasmic vesicles (Fig. 4), some content of putative endocytotic vacuoles (Fig. 7), the matrix of mitochondria, discrete reticulated structures near the plasma membrane (Fig. 6), and some haplosporosomes (Fig. 7) were also stained. Lipoid bodies (Fig. 1), and multivesicular bodies (MVBs) near the parasite surface (Fig. 8), showed varying degrees of ZIO reaction. The matrix of MVB/Golgi complexes, and the dense laminae of confronting cisternae, did not stain with ZIO. Weak labelling of the nuclear membrane, some endoplasmic reticulum (ER), and sER arrays associated with lipid bodies was observed in large dense moribund *Bonamia* sp. in ZIO grids incubated with (control) and without DTT.

Lipids stained most strongly in the lipid bodies (Fig. 9), but also occurred in the membranes, and as irregularly spaced product in the lumina of nuclear membranes (Fig. 9), some (Fig. 10) but not all (Figs. 9 & 11) Golgi cisternae, parallel (Fig. 11) and short (Fig. 12) arrays of sER including those associated with lipid bodies, and in mitochondrial membranes and vesicular cristae (Fig. 12). Lipid deposits were present in the cytoplasm and attached to mitochondrial membranes (Fig. 9), but were particularly apparent within mitochondria (Fig. 12). aER and the surface of some haplosporosomes reacted weakly for lipid (Fig. 12), and membranes in putative phagosomes were moderately to weakly positive (Fig. 13).

Weak β-GPase activity was seen in a few *Bonamia* sp. incubated with and without inhibitors, but was absent from most *Bonamia* sp. and control sections. It was seen in association with membraneous intramitochondrial figures (Fig. 14), and in membraneous vesicles associated with Golgi-like arrays (Fig. 15). Reaction product was not observed within Golgi cisternae, although nuclear membranes and perinuclear Golgi, which are structurally similar (Fig. 16), were well defined in β-GPase-incubated sections (Figs. 15 & 16). β-GPase was most evident in nuclear and mitochon-

drial membranes, short profiles of sER, and some Golgi cisternae of cells incubated with tartrate (Fig. 17).

At pH 5.0, CMPase reaction product was observed in some Golgi cisternae (Fig. 18), in some short ER or aER (Fig. 19) and in association with intramitochondrial membranes. Confronting cisternae showed no enzyme activity (Fig. 18). Coarse reaction product occurred over the nucleolus in some *Bonamia* sp. in sections incubated with and without substrate. Controls and sections incubated with tartrate were negative.

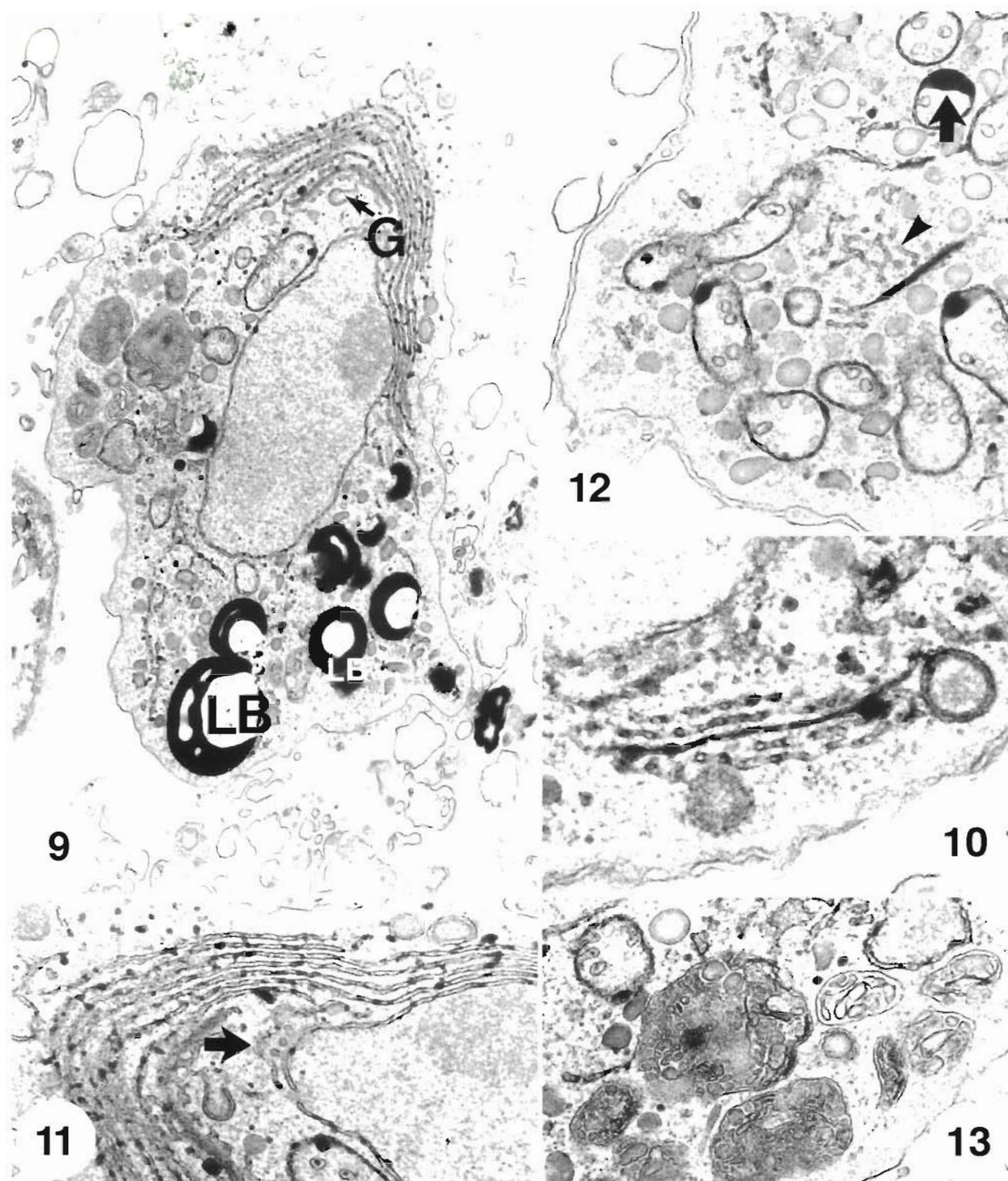
At pH 2.7 CMPase activity was stronger than at pH 5.0, but there was more non-specific reaction product. CMPase 2.7 occurred over the nucleolus, in the nuclear membrane, in Golgi cisternae (Fig. 20), in looped (Fig. 21) and peripheral sER (Fig. 22), and in aER (Fig. 23). Activity was not observed in lipid bodies or in intramitochondrial membranes. Controls were negative except for deposits on the nucleus.

Very few *Bonamia* sp. contained TPPase, and then it occurred very weakly in the lumen of a single Golgi cisterna (Figs. 24 & 25), and in lipid bodies and intramitochondrial membranes of a few parasites. Coarse nuclear deposits were common, especially over the nucleolus (Fig. 24). Occasional observation of intracytoplasmic fine granular deposits was associated with apparent endocytosis of haemocyte TPPase by enclosing phagosome contents using short blunt pseudopodia (Fig. 26). Controls were negative, except for coarse nuclear deposits.

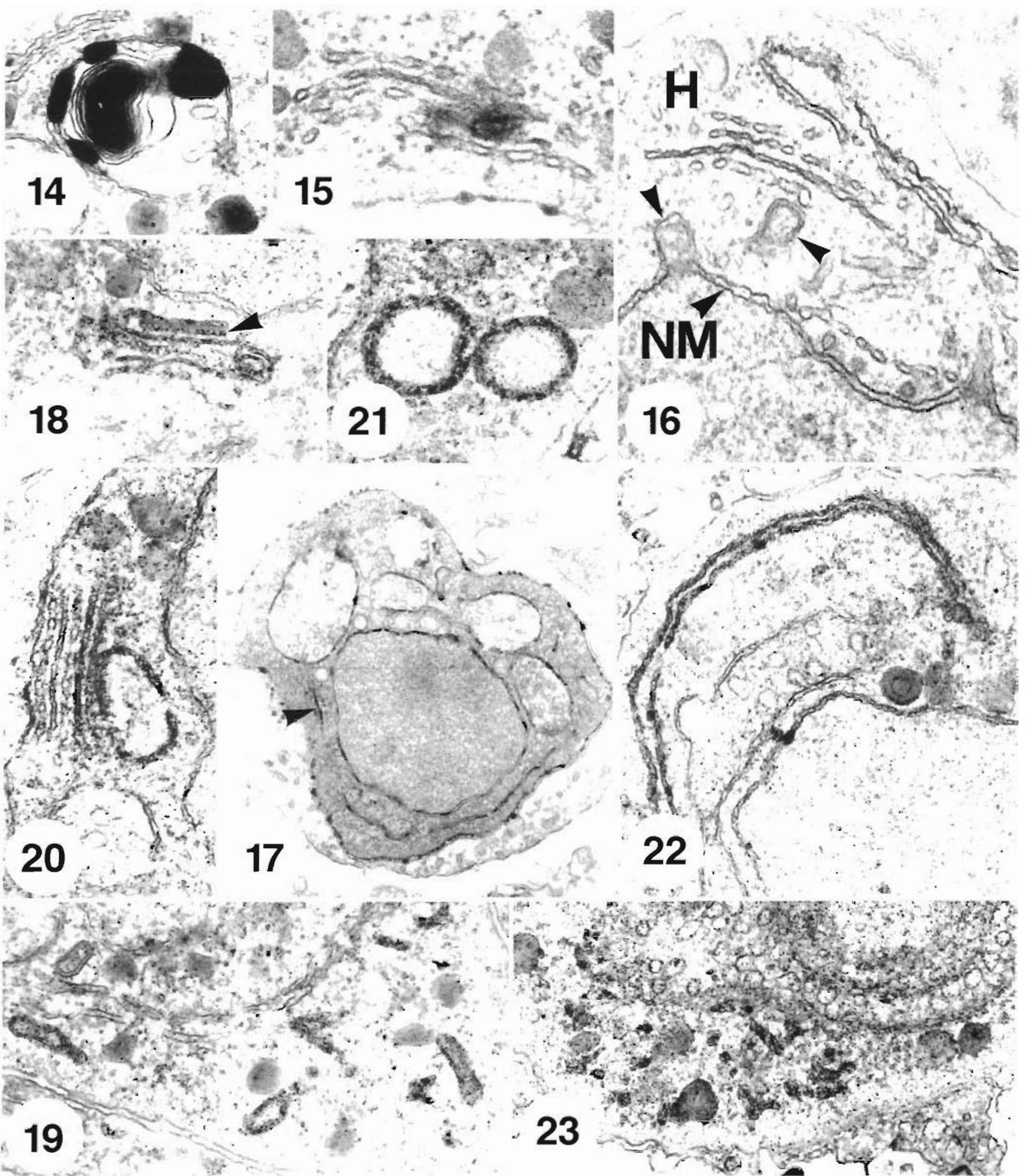
β-Galactosidase was rarely observed, and then only as small intracisternal deposits in Golgi (Fig. 27).

DISCUSSION

The nuclear membrane, sER, Golgi, aER, and MVBs of *Bonamia* sp. were labelled with ZIO, and may be part of an interconnected system, equivalent to the central vacuolar system in mammalian cells (Klausner 1989). The nuclear membrane and sER resemble the same structures in mammalian cells, but there are



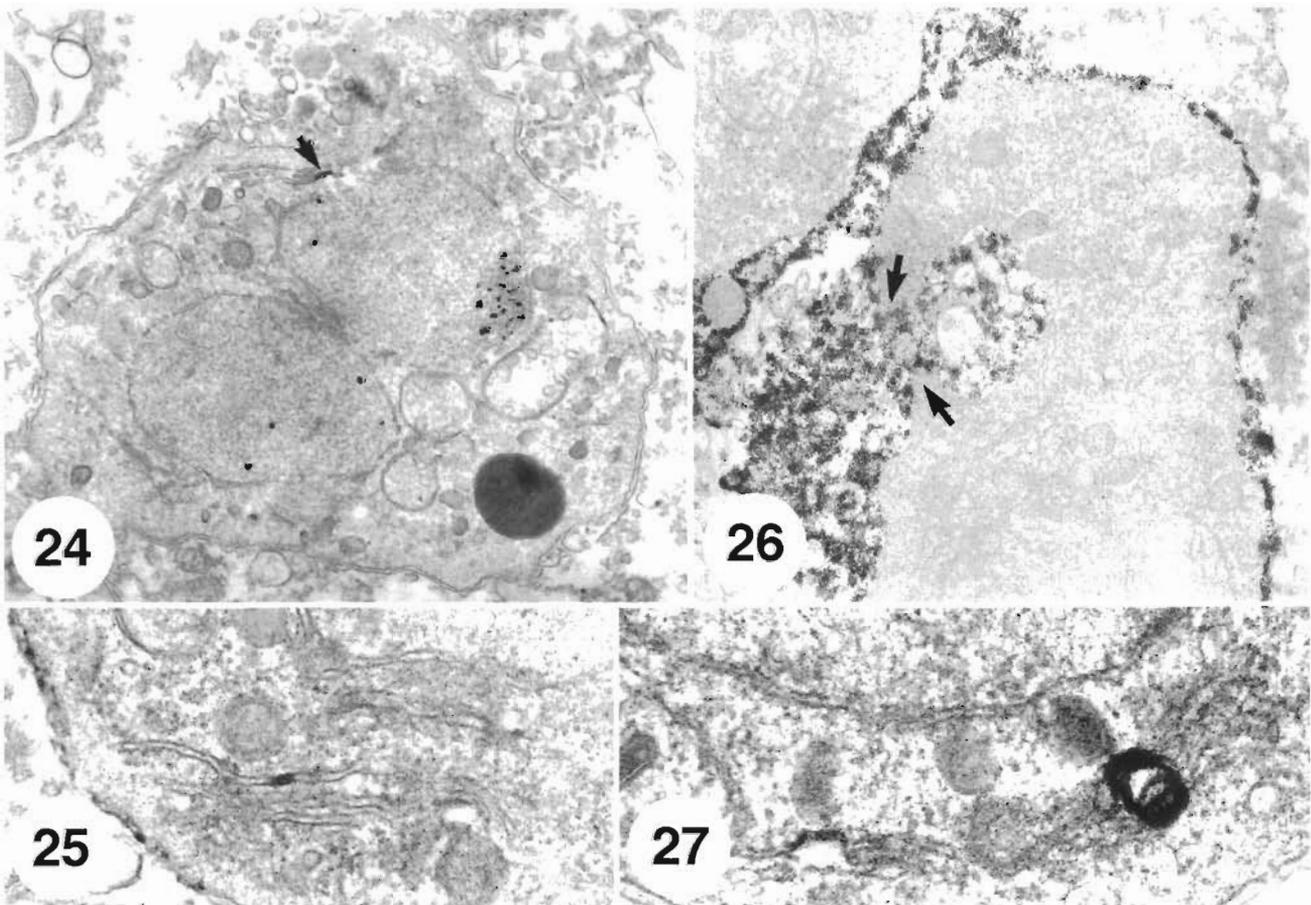
Figs. 9 to 13. *Bonamia* sp. Imidazole (IM) labelling Fig. 9. Late stage *Bonamia* sp showing lipid in the lipid bodies (LB), peripheral sER-like stacks, mitochondria and their membranes, but not in Golgi (G) Pb cit, $\times 20\,130$ Fig. 10 Beaded fenestrated cisternae of perinuclear Golgi with 1 cisterna containing lipid Pb cit, $\times 71\,190$ Fig. 11 Detail of Fig. 9 showing lipid in peripheral Golgi-like sER arrays, but absence from Golgi (arrow). Pb cit, $\times 38\,440$ Fig. 12 Lipid in and on mitochondrial membranes (arrow), in vesicular cristae, and short ER, but only weakly in aER (arrowhead). Pb cit, $\times 45\,000$ Fig. 13 Endocytic vacuoles containing membranes showing moderate to weak reaction for lipid Pb cit, $\times 46\,120$



Figs. 14 to 23. *Bonamia* sp. Acid phosphatases Fig. 14 β -GPase reaction product associated with intra-mitochondrial membranes Pb cit, $\times 54\,070$. Fig. 15. Vesicular β -GPase activity in perinuclear Golgi Pb cit, $\times 96\,260$ Fig. 16 Enhanced membranes of perinuclear Golgi and short ER. Note the similarity in beading in the nuclear membrane (NM) and Golgi cisternae, and the apparent budding of the nuclear surface (arrowheads), possibly to form haplosporosomes (H) β -GPase Pb cit, $\times 67\,780$. Fig. 17. β -GPase-positive nuclear, short ER, and mitochondrial membranes, and positive Golgi cisternae (arrowhead) +ta Pb cit, $\times 23\,000$. Fig. 18. CMPase 5.0-positive perinuclear Golgi cisternae. Note the unreactive CC (arrowhead) Pb cit, $\times 50\,000$. Fig. 19. CMPase 5.0-positive looped ER and aER. Pb cit, $\times 51\,150$ Fig. 20. Perinuclear Golgi showing some positive cisternae CMPase 2.7 Pb cit, $\times 61\,110$. Fig. 21. Positive looped ER. CMPase 2.7 Pb cit, $\times 98\,230$ Fig. 22. Positive peripheral ER. CMPase 2.7 Pb cit, $\times 55\,440$. Fig. 23. Positive aER. CMPase 2.7 Pb cit, $\times 49\,940$

several differences between mammalian Golgi, and the Golgi of *Bonamia* sp. Mammalian Golgi consists of *cis* cisternae that receive vesicles from transitional elements of endoplasmic reticulum (Roth 1989); medial cisternae that contain nicotinamide adenine dinucleotide phosphatase activity (Griffiths & Simons 1986, Roth 1989); and *trans* cisternae which contain TPPase (Griffiths & Simons 1986, Roth 1989, Hermo et al. 1991) and sometimes CMPase (Roth 1989, Hermo et

al. 1991) The *trans* cisternae are connected to a *trans*-tubular or *trans*-Golgi network of anastomosing tubules connected to the *trans* cisternae (Roth 1989), containing TPPase and CMPase (Roth et al. 1985, Hermo et al. 1991) from products which are sorted along various pathways (Griffiths & Simon 1986). Cisternae may be joined by an intercisternal connecting region. The distributions of TPPase and CMPase are variable depending on the cell type and



Figs. 24 to 27. *Bonamia* sp. Fig. 24. TPPase reaction product over the nucleolus and in 1 Golgi cisterna (arrow) Unstained, $\times 22\,480$. Fig. 25. Reaction product in the lumen of a perinuclear Golgi cisterna TPPase Pb cit, $\times 58\,750$. Fig. 26. Haemocyte TPPase-positive material being enclosed by 2 lobopodia (arrows) Unstained, $\times 42\,030$ Fig. 27. β -Galactosidase reaction product in perinuclear Golgi Pb cit, $\times 73\,610$

physiological state of the cell, while lysosomal enzymes may be present throughout the Golgi (Griffiths & Simon 1986).

Two types of Golgi-like structure occur in *Bonamia* sp. Perinuclear cisternae are often connected to the nuclear membrane, comprise 3 to 6 (mean \pm SD = 4.3 ± 0.9 ; $n = 68$) cisternae some of which may appear beaded or fenestrated (Fig. 10), and participate in the processing of putative nuclear material to form haplosporosome-like bodies (Hine 1992, Hine & Wesney 1992). Sometimes the adjacent nuclear membrane is also beaded, and haplosporosome-like bodies appear to bud off from the nuclear membrane (Fig. 16). Perinuclear Golgi differs morphologically from the Golgi of higher animals in the uniformity of cisternae, the absence of vesicles budding from the ends of cisternae, and lack of budding of coated or uncoated vesicles containing protein from *trans* cisternae or *trans*-tubular networks. The second Golgi-like structure comprises large arrays in the peripheral cytoplasm of plasmodial forms (Hine 1991a) that may resemble sER (Figs. 4 & 11) (Hine 1992) or cisternae (Fig. 5) and are often seen enclosing or near lipid bodies (Hine 1992).

Other protists show a range of Golgi morphologies, that range from similarity to the Golgi of higher animals (Queiroz et al. 1991, Vickerman et al. 1991) to endoplasmic reticulum budding coated or uncoated vesicles (Lobo-da-Cunha & Azevedo 1990, Palluault et al. 1990), reticulated tubular structures (Desportes 1976, Lom et al. 1983), or a few small cisternae (Langreth et al. 1978, Kurz & Tiedtke 1993) similar to the perinuclear arrays in *Bonamia* sp. (Hine & Wesney 1992). *Giardia lamblia* forms secretory vesicles from ER in nuclear membrane indentations, as in *Bonamia* sp. perinuclear complexes (Hine & Wesney 1992), but acid phosphatase derives from multiple Golgi-like cisternae (Reiner et al. 1990) similar to arrays in *Bonamia* sp. peripheral cytoplasm (Fig. 5). In *Plasmodium* spp. the ER is dispersed in young stages but forms ordered parallel arrays in older stages (Slomianny & Prensier 1990). Unlike mammalian Golgi, in which the *cis* face stains with ZIO (Parmley et al. 1988), the Golgi of protists (Benchimol & de Souza 1985, Slomianny & Prensier 1990) and protist-like organisms (Palluault et al. 1990) show variable or overall ZIO staining of Golgi. The similar distribution of ZIO reaction in moribund *Bonamia* sp. incubated with (control) and without DTT, suggests DTT does not inhibit ZIO staining, as was also observed in *Pneumocystis carinii* by Palluault et al. (1990).

Apparently *Bonamia* sp. has a system comprising the nuclear membrane, sER, aER, and Golgi-like arrays that may be perinuclear or cytoplasmic, which are involved in lipid and enzyme production and

processing. The system appears to be poorly differentiated morphologically, as perinuclear Golgi appears little modified from the nuclear membrane (Fig. 16) and parallel Golgi-like arrays resemble sER (Fig. 4). There was no evidence of enzyme partitioning or functional polarity in perinuclear Golgi cisternae, and in a previous study (Hine 1992) osmium impregnation did not distinguish a *cis* face, as it does in mammalian cells (Tang & Clermont 1989). Compared with some other protists, the Golgi-endoplasmic reticulum complex appears primitive, both in structure (Vickerman et al. 1991) and function (Langreth & Balber 1975, Queiroz et al. 1991, Kurz & Tiedtke 1993).

Enzymatic activity within protists may depend on activation and the stage of the life-cycle (Reiner et al. 1990, Pimenta et al. 1991), and this must be taken into account when assessing reactivity. Even taking this into account, at normal incubation pH, acid phosphatases (β -GPase, CMPase 5.0, TPPase) and β -galactosidase were often only weakly present or limited in distribution in *Bonamia* sp. CMPase 5.0, TPPase, and β -GPase did not show selective reaction in cisternae of 1 face of Golgi to suggest the existence of *cis* and *trans* faces, although too few observations were made on TPPase to determine such a polarity. It was difficult to assess the degree of reaction of lipid bodies because of their density, but some may have contained very weak CMPase 5.0 and weak to moderate β -GPase.

Several other protists also do not have enzyme content suggesting functional polarity of the Golgi (Pimenta & de Souza 1986, Soares & de Souza 1988, Lobo-da-Cunha & Azevedo 1990, Reiner et al. 1990, Slomianny & Prensier 1990). For example CMPase could not be detected by Palluault et al. (1990) in the Golgi of *Pneumocystis carinii*. However, in *Trypanosoma brucei* the Golgi *trans* face is distinguished by β -GPase (Langreth & Balber 1975), in *Tetrahymena thermophila* the *trans* Golgi is labelled by β -GPase and TPPase (Kurz & Tiedtke 1993), and in *Tritrichomonas foetus* CMPase labels the *trans* face, while TPPase labels the *cis* face (Queiroz et al. 1991). At normal incubation pH the acid phosphatase content of *Bonamia* sp. ER was also weak when compared to other protists (Pimenta & de Souza 1986, Feely & Dyer 1987, Soares & de Souza 1988, Reiner et al. 1990, Slomianny & Prensier 1990).

At pH 2.7, CMPase was observed in the same structures (nuclear membrane, sER, Golgi, aER) as ZIO, which probably comprise a system equivalent to the central vacuolar system of mammalian cells. The intensity of labelling was stronger than for other enzymes, but in the absence of lysosomes or staining of vesicles or lipid bodies, there was no apparent storage of the enzyme. Although Hervio et al. (1991) suggested acid

phosphatase may be used to coat and protect the plasma membrane as in *Leishmania* spp., there was no evidence of an acid phosphatase surface coat herein. The substance coating *Leishmania* spp. has now been identified as lipophosphoglycan (LPG) which is formed from acid phosphatase in the Golgi by the addition of a carbohydrate structure (Bates et al. 1990).

Bonamia sp. grows and divides inside haemocytes, and therefore it must endocytose material. As *Bonamia* sp. in parasitophorous vacuoles (PVs) are often surrounded by membraneous particles, the MVBs observed in *Bonamia* sp. in this (Figs. 8 & 9) and a previous study (Figs. 22 & 23 in Hine & Wesney 1992) may result from pinocytosis or phagocytosis. Ultrastructurally the MVBs of *Bonamia* sp. resemble the MVBs of *Trypanosoma cruzi* that form from fusion of pinocytotic vesicles and which are involved in protein uptake (de Souza et al. 1978, Soares & de Souza 1988). If the MVBs in Figs. 22 & 23 of Hine & Wesney (1992) do result from endocytosis, the close apposition of MVBs and Golgi in Fig. 23 suggests that Golgi-derived vesicles containing hydrolytic enzymes may fuse directly with endocytotic MVBs, rather than first forming a lysosome that then fuses with the MVB. Some acid phosphatase may be stored in lipid bodies, but there was no apparent interaction between endocytotic MVBs and lipid bodies.

Intense IM staining of the lipid bodies indicates that this organelle, which lacks a bounding membrane, has a high lipid content. The presence of lipid in the lumina of the nuclear membranes, some perinuclear Golgi cisternae, and ER suggests that lipid is processed through these compartments, although aER appeared to be less involved in lipid processing. There was a close association between lipid-rich sER arrays (Fig. 11) and lipid bodies (Fig. 5), similar to the association between ER arrays and lipid bodies in *Leishmania* (Pimenta & de Souza 1986) and other trypanosomatids (Soares & de Souza 1988). Staining with IM also indicated that mitochondria may act as a centre for lipid synthesis or storage, as in some other protists (Zierdt et al. 1988).

Lipid bodies may function in 1 or more of 4 different ways. (1) They may be storage organelles for excess triglycerides. As IM binds to unsaturated lipids such as triglycerides (Angermüller & Fahimi 1982), lipid bodies probably have a high unsaturated lipid content. During the annual infection cycle of the parasite in oysters (Hine 1991a, b) *Bonamia* sp. proliferates and grows (Hine 1991a) in haemocytes that re-absorb unspawned oyster ova (Hine 1991b). Oyster ova are rich in triglycerides (Lee & Heffernan 1991) and at the time of ova reabsorption lipid bodies and large sER arrays develop in the parasite (Hine 1991a). Lipid bodies may therefore be stores of triglycerides derived

from host ova and other cells and may be an energy reserve for the parasite.

(2) Lipid bodies may be involved in lipid secretion or excretion. In phagocytosis, *Bonamia ostreae* suppresses the haemocyte respiratory burst (Hervio 1992) and, like *Bonamia* sp., comes to lie in a PV. Some api-complexan protozoans that utilize phagocytes discharge lipid-like vesicles that enlarge the phagosome membrane to produce a PV, and modify it to prevent lysosome-phagosome fusion (Sibley 1989). Thus lipid bodies in *Bonamia* spp. may discharge similar vesicles to modify the host phagosome membrane, form the PV, and prevent lysosome-phagosome fusion.

(3) Contrary to a previous study (Hine 1992), lipid bodies appeared to contain some β -GPase, occasional very weak TPPase, and possibly some CMPase 5.0. Hervio et al. (1991) observed CMPase in lipoidal bodies of *Bonamia ostreae*. In *Leishmania mexicana amazonensis* the ER around lipid bodies contains β -GPase (Fig. 5 in Pimenta & de Souza 1986). The lipoidal body may therefore be a lipolysosome that participates in cellular defence or digestion.

(4) Unsaturated lipids may, as in *Leishmania* spp., scavenge H_2O_2 generated as a killing mechanism by host haemocytes (Channon & Blackwell 1985).

The main function of lipid bodies is probably lipid metabolism based on triglycerides, as lipid metabolism may explain both the presence of confronting cisternae (CC) and cylindrical confronting cisternae (CCC) in some *Bonamia* late in the infection cycle (Hine & Wesney 1992), and the weak hydrolytic enzymes. Unusual membraneous structures, including some resembling CCC, are formed in fish enterocytes involved in lipid metabolism (Ostos Garrido et al. 1993). In vertebrates, lipids are hydrolysed extracellularly by pancreatic lipase, combine with bile to form micelles that diffuse through the plasma membrane, and are re-synthesised intracellularly as triglycerides. Haemocytes may hydrolyse triglyceride-rich cytosol from ova before or after endocytosis, making breakdown products directly available to the parasite. Direct utilisation of fatty acids and glycerol from haemocytes, and their re-synthesis into triglycerides within the parasite, would negate the need for a well-developed lysosomal system storing hydrolytic enzymes in *Bonamia* sp., and would involve mitochondria in triglyceride re-synthesis, as suggested from enzyme and IM reaction observed herein. Utilisation of lipids by the parasite would also explain the lack of intracellular glycogen or other carbohydrate reserve. The presence of CC, CCC, haplosporosome-like bodies, and bizarre membraneous configurations in *Bonamia* sp. late in the annual infection cycle (Hine & Wesney 1992) may therefore be attributable to breakdown of lipid metabolism due to parasite senescence.

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