Cell Fractionation of Parasitic Protozoa – A Review

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Cell fractionation, a methodological strategy for obtaining purified organelle preparations, has been applied successfully to parasitic protozoa by a number of investigators. Here we present and discuss the work of several groups that have obtained highly purified subcellular fractions from trypanosomatids, Apicomplexa and trichomonads, and whose work have added substantially to our knowledge of the cell biology of these parasites.

Key words: parasitic protozoa - cell fractionation - trypanosomatids - Apicomplexa - trichomonads

The protozoa comprise a large number of species, including some agents of human and animal diseases such as malaria, leishmaniasis, Chagas disease, African trypanosomiasis, amebiasis, giardiasis, toxoplasmosis, coccidiosis, theileriosis, and babesiosis, to mention only some of the more important examples. These protozoa are also of interest from a cell biological point of view since they contain unique cytoplasmic structures and organelles that have been the subject of investigation in some detail for many years. These studies have provided new information of general biological interest since some investigators assume that some of the protozoa form the roots of the evolutionary tree of eukaryotic cells.

THE CELL FRACTIONATION TECHNIQUE

To disrupt tissue cells, separate their components by differential centrifugation and analyze the distribution of known enzymes among the different fractions obtained has been a powerful tool for exploring cell structure and composition.

Transmission electron microscopy of thin sections has provided detailed views of cell structure. However, to address many questions concerning the function of subcellular organelles it has proven necessary to gently disrupt the cell and isolate its components. This has been accomplished by differential centrifugation, a methodology developed in the 1940s and 1950s mainly by the cell biology group coordinated by Albert Claude, Christian de Duve and George Palade at the Rockefeller University. Their reviews should be consulted for descriptions of the early studies (De Duve 1971, 1975, Claude 1975, Palade 1975 De Duve & Beaufay 1981). Their work established a baseline of important concepts: (a) polydispersity, which allowed the rules of sedimentation coefficients (Svedberg & Pedersen 1940), as defined for macromolecules, to be adapted for subcellular particle centrifugation; (b) biochemical homogeneity and unique localization, which resulted in the definition of marker enzymes; and (c) latency, which distinguished among enzymes confined in different membrane-bound compartments by the differences in time of release after membrane lyses (De Duve 1967). Subsequently, the knowledge obtained by analytical cell fractionation has been used to design preparative cell fractionation schemes aimed at isolating and purifying specific organelles for further studies.

The cell fractionation process usually starts with disruption of the plasma membrane, using conditions that minimize damage to the membranes bounding intracellular organelles. Disruption can be achieved by procedures such as exposure to high-frequency sound (sonication), treatment with a high-speed blender, grinding in a mechanical homogenizer, nitrogen cavitation, etc. Usually, it is necessary to monitor the process by phase contrast microscopy in order to avoid rupture of the organelles or disorganization of cytoplasmic structures. Once the cells are broken open, the suspension can be separated into its main components using a series of centrifugations at increasing speeds. This causes cell components to move toward the bottom of the centrifuge tube, forming a pellet at a rate that depends on their size and density. The supernatant is then collected and subjected to further centrifugation at higher speed, and the process may be repeated several times depending on the cell type and the structures to be isolated. Usually differential centrifugation does not yield very pure material. Therefore, it is necessary to continue the isolation procedure by using density-gradient centrifugation, a procedure where the organelles and structures are separated by sedimentation through a gradient of a dense solution such as sucrose, Metrizamide, Percoll, etc. In this process, the fraction first obtained by differential centrifugation is layered on the top of a gradient composed of a non-ionic substance. Alternatively, sample can be positioned under the gradient solution or mixed with it, in the case of self-forming gradients. The tube is centrifuged at high speed for a extended period, allowing each structure to migrate to an equilibrium position where the density of the structure is equal to that of the surrounding liquid. Therefore, in this process separation is based on the buoyant density rather than on the size and shape of the particles. Following centrifugation, the fractions can be collected individually for further analysis and determination of the efficiency of separation.

An important requirement for successful cell fractionation is the evaluation of the isolation procedure, usually accomplished using morphological and/or biochemical methods. The morphological approach applies light and electron microscopy of thin section to the various fractions obtained. The biochemical approach is based on the determination of the enrichment of enzymes or other molecules (a special type of lipid, an antigen, etc.) that can be considered as a marker for a given structure or organelle. To be considered a marker, an enzyme must be known as a component of the organelle in all cells studied (biochemical homogeneity) and absent from the rest of the cell (unique localization). These notions resulted from analytical cell fractionation studies (De Duve 1975) and defined a number of classical enzyme markers (Table). Based on experience accumulated over many years, we strongly recommend the simultaneous use of both approaches.

There are relatively few reports on the isolation of structures and organelles from parasitic protozoa due to several factors, including difficulty in obtaining enough cells to start the fractionation procedure, and difficulty in breaking open the cells under conditions in which the structures are well preserved. This is especially important for those protozoa in which a layer of subpellicular microtubules renders the cells more resistant to rupture, as in the case of trypanosomatids and Apicomplexa. Finally, there is the absence of well-defined markers to characterize the isolated structure.   Nevertheless, the discovery of two special organelles from protozoa came from analytical cell fractionation experiments: the glycosome of trypanosomatids (Opperdoes & Borst 1977) and the hydrogenosome of trichomonads (Lindmark & Muller 1973).

The present review will describe attempts made to obtain structures and organelles from protozoa belonging to the Trypanosomatidae and Trichomonadidae families and the Apicomplexa phylum. They were selected because they are good examples of the use of the cell fractionation technique to obtain highly purified and well characterized fractions, which were further characterized using morphological and biochemical approaches.

CELL FRACTIONATION OF TRYPANOSOMATIDS

Fig. 1 shows a general view of a thin section of a trypanosomatid examined by transmission electron microscopy. The protozoan is surrounded by a typical plasma membrane, which envelops the cell body, invaginates at the anterior end of the cell forming the flagellar pocket, and continues over the flagellum. Therefore, we can distinguish at least three main domains of the plasma membrane: those surrounding the cell body, the flagellum, and the lining of the flagellar pocket. Below the plasma membrane, except in the region of the flagellar pocket, there is a layer of microtubules, known as subpellicular microtubules, which are in contact with each other, with the plasma membrane and with profiles of the endoplasmic reticulum through filamentous structures. The flagellum emerges from the basal body located in the anterior region of the cell and projects forward. It displays a typical flagellar structure with a 9+2 pattern of microtubule doublets. In addition, it exhibits a highly elaborated network of filamentous structures connected to the axoneme through small bridges forming a structure known as the paraflagellar (or paraxial) structure (or rod). The nucleus of the protozoan is centrally located. The cytoplasm contains randomly distributed ribosomes and profiles of endoplasmic reticulum. The Golgi complex is located in the anterior region, close to the flagellar pocket. Some organelles are specific for the trypanosomatids. The first one is the kinetoplast, which corresponds to a condensation of extranuclear DNA that lies within the unique ramified mitochondrion, and is localized in front of the basal body that gives rise to the flagellum. The second characteristic organelle, designated the glycosome, is usually round, is enveloped by a unit membrane, and contains a matrix denser than the cytoplasm. Most of the enzymes of the glycolytic pathway are localized in this organelle. Since in some species the glycosome contains catalase and en-

TABLE

Enzymes recognized as markers of cell organelles in mammal cells and protozoa

Organelle	Marker enzyme	
	Mammal cells	Protozoa
Plasma membrane	5'-nucleotidase Na$^+$-K$^+$-ATPase adenyl cyclase	5'-nucleotidase [a] Na$^+$-K$^+$-ATPase [a] Mg^{2+}-ATPase adenyl cyclase
Mitochondria	cytochrome oxidase	succinate-cytochrome c reductase
Endoplasmic reticulum	glucose-6-phosphatase	glucose-6-phosphatase NAPH-cytochrome c reductase
Golgi complex	sialyl transferase	galactosyl transferase
Glycosome (trypanosomatids)	not present	hexokinase
Hydrogenosome (trichomonads)	not present	malic decarboxylase
Acidocalcisome	not present	H$^+$ pyrophosphatase

a: in *Tritrichomonas foetus*

zymes involved in the β-oxidation of lipids, it corresponds to a peroxisome. The third characteristic organelle consists of several distinctive vesicles, which usually display an empty interior and an electron-dense material at their periphery. This organelle, designated acidocalcisome, has been the subject of intense investigation in recent years and is involved in the regulation of the cytoplasmic concentration of calcium, with the participation of several ATPases. Another important organelle is the reservosome, which is found in epimastigote forms of trypanosomatids of the genus *Trypanosoma*, of the subgenus *Schyzotrypanum*, and is involved in the accumulation of macromolecules ingested through the endocytic pathway.

Isolation of the plasma membrane

Attempts have been made to obtain a pure plasma membrane fraction from trypanosomatids. This is not an easy task, as the continuous layer of microtubules below the plasma membrane makes trypanosomatids resistant to conventional methods for cell breakage. Hunt and Ellar (1974) disrupted *Leptomonas collosoma* by blending the cell suspension with glass beads and were able to isolate a plasma membrane fraction. They found that the subpellicular microtubules remained attached to the plasma membrane, and could be used as a morphological criterion in assessing the purity of the fraction. This association of isolated membranes with microtubules has been confirmed for *L. collosoma* (Linder & Staehelin 1977), *Trypanosoma cruzi* (Meyer & De Souza 1976), *Trypanosoma brucei* (Voorheis et al. 1979), *Leishmania mexicana* (Timm et al. 1980), *Leishmania donovani* (Dwyer 1980), and *Trypanosoma lewisi* (Dywer & D'Alessandro 1980). In some cases, the microtubules did not remain associated with the plasma membrane (Pereira et al. 1978, Franco da Silveira et al. 1979, Zingales et al. 1979, Rovis & Baekkeskov 1980). In these cases the only way to assess the purity of the fraction is to use the membrane enzyme markers or the attachment of microtubules to the isolated membranes. Since different methods have been used to rupture the cells, we cannot exclude the possibility that, in some cases, the attachment of microtubules to the membrane depends on differences in the methods used. But even when the same method was used for the isolation of the membrane, it gave different results in *T. cruzi* (Pereira et al. 1978) and in *L. mexicana* (Timm et al. 1980).

Three procedures have been used to obtain a purified plasma membrane fraction from epimastigotes of *T. cruzi*. Cells previously swollen by treatment with Triton X-100 were disrupted using a Dounce-type homogenizer. The membrane fraction was obtained through successive steps of differential centrifugation and its purity was evaluated by electron microscopy and by testing for enzyme markers. Activities of 5'-nucleotidase and Na$^+$-K$^+$-ATPase, the two classical markers of the plasma membrane in mammals, were not found in *T. cruzi*. Acid phosphatase and succinate dehydrogenase were absent from the purified membrane fraction. The specific activities of Mg^{2+}-dependent ATPase and adenyl cyclase in the membrane fraction were two and five times higher, respectively, than in the whole homogenate. Electron-microscopy

showed slight contamination with ribosomes (Timm et al. 1980).

In the second approach, cells were ruptured by sonication and the membrane fraction were isolated by differential centrifugation, followed by equilibrium centrifugation on sucrose gradients. The fractions were characterized by electron microscopy, enzyme markers (adenyl cyclase, succinate dehydrogenase, cytochrome c reductase, acid phosphatase, and glucose-6-phosphatase), and by distribution of surface-bound ^{131}I activity in cells previously labelled using lactoperoxidase. A 10-fold increase in the adenyl cyclase activity was obtained with this method. Na$^+$-K$^+$-ATPase and 5' nucleotidase activities were not found (Franco da Silveira & Colli 1981). In a third report, plasma membrane was induced to vesiculate by incubating the cells with aldehydes, N-ethylmaleimide, p-chloromercuribenzoate, or acid buffers, followed by isolation of the vesicles by sucrose-density centrifugation (Franco da Silveira et al. 1979). The fractions were characterized by distribution of ^{131}I activity as in the second approach described above, and by electron microscopy. Unfortunately, the authors did not assay enzyme markers.

Examination of the electron micrographs of *T. cruzi* treated with vesiculating agents showed that vesicles form only at certain points of the membrane which encloses the cell body and the flagellum. Therefore, the membrane fraction isolated by this procedure certainly does not contain all areas of the plasma membrane. The method is interesting as it shows that we can examine specific regions of the cell surface of *T. cruzi* that, for unknown reasons, are more likely to form vesicles. A 30-fold to 50-fold enrichment of some proteins was detected in the membrane fraction isolated from the vesicles (Franco da Silveira et al. 1979).

These studies indicate that the plasma membrane of epimastigotes of *T. cruzi* does not have two of the enzymes that are typical of the plasma membrane of eukaryotic cells: Na$^+$-K$^+$-ATPase and 5'-nucleotidase. It seems that adenyl cyclase is the best marker to determine the purity of a plasma membrane fraction of *T. cruzi*. In *T. brucei*, Na$^+$-K$^+$-ATPase was found and was used to establish the purity of the isolated plasma membrane fraction (Rovis & Baekkeskov 1980, Mancini et al. 1982).

Three procedures have been used to obtain a plasma membrane fraction from *T. brucei*. First, cells were disrupted using nitrogen cavitation followed by differential centrifugation and successive centrifugations in gradients of dextran and sucrose (Rovis & Baekkeskov 1980). The fraction obtained was characterized by electron microscopy and by using enzyme markers (Na$^+$-K$^+$-ATPase and adenyl cyclase) and polyacrylamide gel electrophoresis. In the second approach, cells were broken by sonication, followed by differential centrifugation and centrifugation in a sucrose gradient (Mancini et al. 1982). The fraction obtained was characterized by electron microscopy, assay of enzyme markers (adenyl cyclase and Na$^+$-K$^+$ATPase), analysis of iodinatable surface proteins, and the presence of the variable surface coat glycoprotein. Finally, cells were disrupted using a Dounce homogenizer followed by centrifugation in a sucrose gradient.

The fraction obtained was characterized by electron microscopy and assay of the enzyme markers Na$^+$-K$^+$-ATPase and adenyl cyclase (Voorheis et al. 1979).

L. donovani cells, previously treated with dilute ethanol in a hypotonic buffer, were disrupted on ice in a Dounce homogenizer and the membrane fraction was purified by centrifugation in a sucrose gradient. Characterized by electron microscopy, the fraction exhibited plasma membrane profiles with associated subpellicular microtubules (Fig. 2), which served as natural morphological markers to distinguish the plasma membrane from internal membranes profiles (Dwyer 1980).

The freeze-fracture technique (reviewed in De Souza 1995) shows that the membrane that covers the various regions of the trypanosomatids (cell body, flagellar pocket and flagellum) is not homogeneous. Therefore, it would be of interest to obtain membrane fractions from each of the different regions. Some attempts have been made to isolate the flagellar membrane. On treating an isolated flagellar fraction of *T. cruzi* with Triton X-100, Piras and co-workers (1981) obtained a soluble flagellar membrane preparation, but did not characterize it in detail. Some years later, Cunha-e-Silva and co-workers (1989) isolated a highly purified flagellar membrane fraction from two different species of trypanosomatids. By treating a flagellar fraction devoid of cell bodies with Triton X-100 we separated the flagellar membrane by centrifugation in a sucrose gradient (Fig. 4; see the following section).

Reviewing the work on the isolation of the plasma membrane of trypanosomatids has led to the important conclusion that the bridges of a protein nature connecting the inner portion of the plasma membrane to the subpellicular microtubules showed by Souto-Padrón and co-workers (1984) in quick-frozen and deep-etched replicas of *T. cruzi* are very stable structures.

Isolation of the flagellum

The flagellum is responsible for the motility of trypanosomatids and participates in their interaction with the hosts, by adhering to the insect digestive tract (Vickerman & Tetley 1990), or initiating the contact with mammalian cells (De Souza 1984). It arises from a basal body at the bottom of a plasma membrane invagination, the flagellar pocket. The intraflagellar cytoskeleton comprises a canonical type 9+2 axoneme and a unique paraflagellar or paraxial structure, which is present in all trypanosomatids except for those that bear an endosymbiont (Freymuller & Camargo 1981).

The initial attempts to isolate the flagellum of trypanosomatids were published in 1977. Segura and co-workers (1977) disrupted *T. cruzi* epimastigotes using cavitation. The flagellum-enriched fraction obtained by differential centrifugation was contaminated with membranes of flagellar and non-flagellar origins, due to the cell rupture method. The authors performed double diffusion tests against an antibody raised to the entire parasite and found five precipitin lines against the flagellar fraction. Flagella also showed good results (80 to 90%) in protective studies against a lethal challenge with trypomastigotes. The use of adjuvants raised this protection to 100% (Ruiz et al. 1986). It is noteworthy that the flagellar was the only

subcellular fraction that did not cause cardiac lesions when inoculated into mice (Ruiz et al. 1986). In the same year, Pereira and co-workers (1977) designed another method for isolating flagella based on separating the flagellum from the undisrupted cell body. They treated *Crithidia fasciculata, Herpetomonas samuelpessoai* and *Leishmania tarentolae* with Lubrol-PX, a non-ionic detergent, in the presence of magnesium chloride, before deflagellation with a Dounce homogenizer. Flagellar fractions thus obtained were very pure, but consisted of demembranated flagella as a result of the detergent. Although devoid of membranes, isolated flagella from *H. samuelpessoai* were very effective in protecting mice against *T. cruzi*, indicating that highly conserved antigens were present inside the flagella. One year later, Pereira and co-workers (1978) obtained a similar flagellar fraction from *T. cruzi* epimastigotes. This fraction was also protective.

In 1981, Piras and co-workers developed a protocol for the isolation of the flagellum of *T. cruzi* epimastigotes, based on sonication of the parasites in the presence of bovine serum albumin and calcium chloride to protect the cell body from rupture, followed by differential and sucrose gradient centrifugation. They obtained a highly purified and well-preserved flagellar fraction. By treating the flagellar fraction with Triton X-100, they also isolated an axoneme (plus paraxial rod) fraction and a soluble flagellar membrane fraction. Both flagellar cytoskeleton and flagellar membranes gave precipitin lines in double-diffusion tests against chagasic patients' sera.

We have adapted and improved Piras' method to purify flagella from *H. megaseliae* (Cunha et al. 1984). This monoxenic trypanosomatid possesses a very big paraxial rod. The flagellar fraction from *H. megaseliae* was highly purified and we demonstrated by electron microscopy that the flagella were well preserved and had intact membranes (Fig. 3). In the same study, we obtained purified flagella from *Crithidia deanei,* a trypanosomatid that does not possess the paraxial structure. By comparing the electrophoretic profiles of purified flagella from both parasites, we could identify which protein were the major components of the paraxial structure of *H. megaseliae.*

Since the first protection studies with flagellar fractions (see above), it was clear that an internal component of flagella (even from monogenetic trypanosomatid species) could confer a high degree of protection against lethal challenges with *T. cruzi* trypomastigotes (Segura et al. 1974, 1977, Pereira et al. 1977, Ruiz et al. 1985). Considering that the components of the axoneme are highly conserved in eukaryotes, the paraflagellar structure had the potential to be used as an immunogen for vaccination, with the advantage that it is different from any cytoskeletal structure of the mammalian host cell. Pursuing this lead, a research group developed immunization protocols using purified major paraxial proteins with excellent results (Segura et al. 1974, Wrightsman et al. 1995, Miller et al. 1996, 1997).

The paraxial structure is an intricate lattice of protein filaments that parallels the axoneme for almost all its length and is connected to its microtubule doublets 4 to 7 (Fuge 1969, De Souza & Souto-Padrón 1980). Three portions

form the lattice: one proximal to the axoneme, one distal and one intermediary portion connecting the other two. Layers of thin (7 nm) and thick (25 nm) filaments crossing at an angle of 100º form the basic organization of the proximal (two layers) and distal (eleven layers) portions. Alternating Y- and I-shaped thin filaments form the intermediary portion (Farina et al. 1986). The biochemical com-

position of the paraxial structure was analyzed by different approaches and shown to contain two major proteins in all trypanosomatids: a slowly migrating protein, PFR1, ranging from 70 to 78 kDa and a faster one, PFR2, ranging from 68 to 73 kDa (reviewed in Bastin et al. 1996). It was recently demonstrated that at least in *T. cruzi* there are four major proteins, two of 70 kDa – PAR1 and PAR 3 –
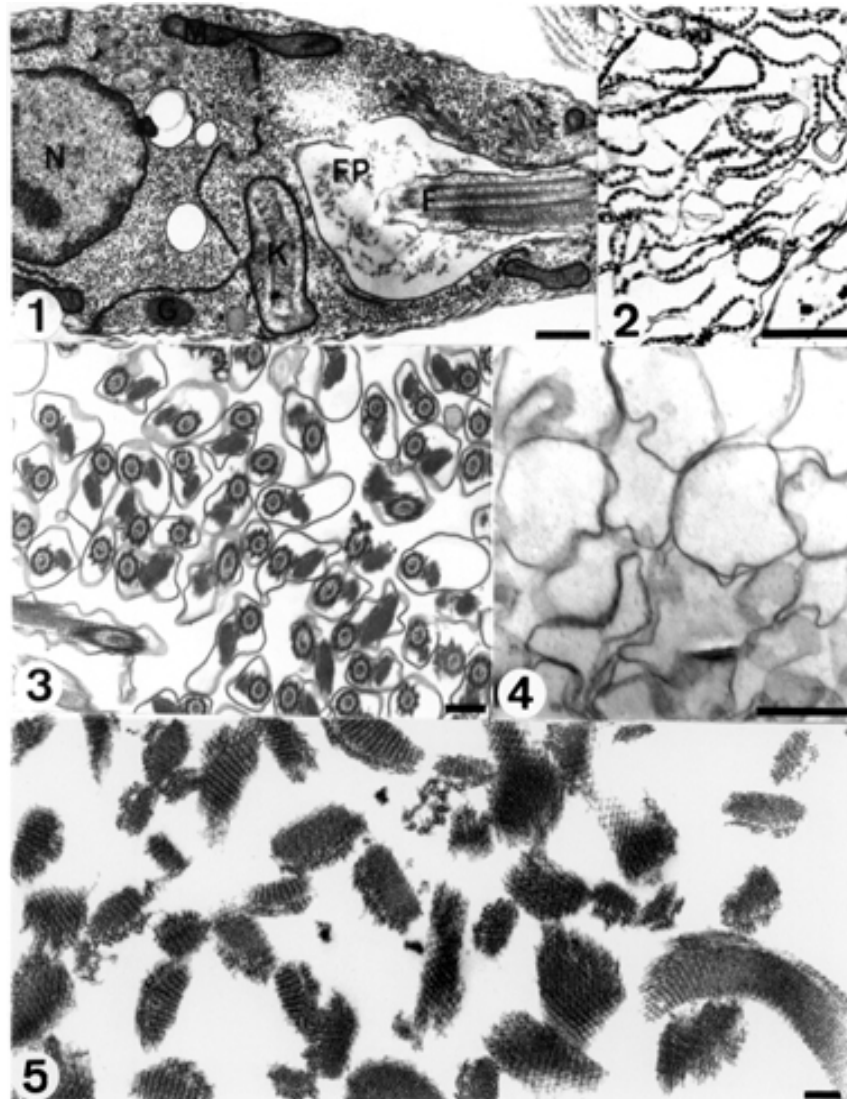

Fig. 1: longitudinal section of *Herpetomonas anglusteri* showing structures such as the flagellum (F), the nucleus (N), the kinetoplast (K), mitochondria (M), glycosomes (G) and the flagellar pocket (FP) (after Motta et al. 1991). Bar = 0.5 µm. Fig. 2: a pure fraction containing plasma membranes of *Leishmania donovani* promastigotes is characterized by the presence of closely associated subpellicular microtubules (after Dwyer 1980). Bar = 0.5 µm. Fig. 3: purified flagellar fraction from *Herpetomonas megaseliae* (after Cunha et al. 1984). Bar = 0.5 µm. Fig. 4: highly purified flagellar membrane fraction from *Trypanosoma cruzi* (after Cunha-e-Silva et al. 1989). Bar = 0.5 µm. Fig. 5: purified paraflagellar rod fraction from *H. megaseliae* (after Moreira-Leite et al. 1999). Bar = 0.1 µm

and two of 68 kDa – PAR2 and PAR4 (Fouts et al. 1998).

There is no correlation between paraxial major proteins and paraxial filaments. Furthermore, it is evident that such an intricate structure must have minor components. For *T. brucei*, those minor components were suggested to be spectrin-type proteins (Schneider et al. 1988, Woods et al. 1989, Müller et al. 1992, Woodward et al. 1994, Imboden et al. 1995).

The best way of investigating the paraflagellar rod composition was to obtain a subcellular fraction containing purified and intact paraxial rods. We decided to try to improve a protocol described by Russell and co-workers (1983), who used trypsin to disrupt the connections between paraxial rod and axoneme. Purified and detergent-demembranated flagella from *H. megaseliae* were treated with trypsin in a very careful manner and applied on top of a 1.8-2.2 M continuous sucrose gradient. After centrifugation, six fractions of equal volume were collected from top to bottom. The third fraction was a highly purified paraflagellar rod fraction (Fig. 5) (Moreira-Leite et al. 1999). Using tubulin as an internal marker it was demonstrated by SDS-PAGE and Western blots that the fraction was devoid of axonemes. Fractions from higher sucrose densities were progressively richer in axonemes. The purified paraflagellar fraction allowed us to identify minor components in the range of 120 to 190 kDa.

The flagellar membrane is also of great interest, as many trypanosomatids adhere to their insect hosts by the flagellum (Steiger 1973, Vickerman 1973, reviewed in De Souza 1989) and depend on this adhesion to survive and differentiate. Ultrastructural studies have demonstrated some interesting features concerning the flagellar membrane: freeze-fracture replicas of *L. samueli, H. megaseliae* and *T. cruzi*, (Souto-Padrón et al. 1980, 1984) showed that the membrane that surrounds the flagellum has far fewer intramembranous particles than the membrane enclosing the cell body of the same cell, suggesting a low protein/lipid ratio. Special particle arrays are present at the membrane of the flagellar base and flagellum-cell body attachment region (Linder & Staehelin 1977, De Souza et al. 1978b, 1979). Although poor in protein, flagellar membrane is immunogenic, since it is recognized by chagasic serum (see above, Piras et al. 1981).

We managed to get a highly purified flagellar membrane fraction from *H. megaseliae* and *T. cruzi* epimastigotes (Fig. 4) (Cunha-e-Silva et al. 1989) by combining detergent treatment on ice, vortexing and separation on a sucrose equilibrium gradient. Analyzing the purified fraction by using filipin cytochemistry for sterols combined with freeze-fracture, we showed that the flagellar membrane is almost devoid of intramembranous particles and rich in sterols. Electrophoretic analysis showed few bands, some of which bound Concanavalin A after being transferred to nitrocellulose. The identification of these protein bands as receptors or adhesins still awaits further investigation.

Isolation of the mitochondrion-kinetoplast complex

Trypanosomatids possess only one ramified mitochondrion, which spans the parasite's entire body. In the portion of the mitochondrion localized below the basal body, where the flagellum arises, there is a special concentration of DNA molecules that form a slightly concave disc-like structure, the kinetoplast. Kinetoplast DNA is composed of about 25,000 concatenated minicircles and about 30 maxicircles in a single network per cell and represents up to 25% of the total DNA. It would be important to obtain a purified subcellular fraction containing the mitochondrion-kinetoplast complex, as it would allow studies concerning the molecular characteristics of kinetoplast DNA and its processing, and a better understanding of parasite metabolism, including the balance between aerobic and anaerobic metabolism in bloodstream and procyclic forms of *T. brucei*.

Hill and co-workers (1968) obtained the first mitochondrion-kinetoplast enriched fraction from *C. fasciculata* and characterized the cytochromes from this protozoan. Braly and co-workers (1974) isolated the mitochondrion-kinetoplast complex from *L. tarentolae,* obtaining a purified fraction, but the mitochondrion was fragmented.

Nichols and Cross (1977) used digitonin under hypotonic conditions to disrupt *C. fasciculata*, and purified the mitochondrion-kinetoplast complex using differential centrifugation and density gradients, analyzing its biochemical properties and RNA content. Another mitochondrial fraction from *C. fasciculata* was prepared at the same time by Edwards and Lloyd (1977) using rate zonal centrifugation. By evaluating the activity of the enzymes NADH-cytochrome c oxidoreductase and p-nitrophenyl phosphatase in each fraction, they demonstrated the separation of mitochondria from lysosomal vacuoles.

Several years later, Opperdoes and co-workers (1981) established a cell fractionation method for procyclic forms of *T. brucei*. They obtained a glycosome fraction equilibrating at 1.23 g/cm^3 and a mitochondrial fraction equilibrating at 1.16 to 1.18 g/cm^3. They showed the presence in the latter of succinate dehydrogenase and oligomycin-sensitive ATPase activity as well as L-threonine 3-dehydrogenase and carnitine acetyl transferase. The latter two enzymes, involved in threonine metabolism, were 15-fold higher in procyclic mitochondrial fraction than in that of bloodstream forms.

Cell disruption using silicon carbide and grinding in a mortar was applied to *T. cruzi,* followed by isopycnic centrifugation, in order to determine the subcellular localization of phosphoenolpyruvate carboxykinase and malic enzyme I (Cannata et al. 1982). They were found in two different compartments, glycosomes and mitochondrion, respectively.

Subcellular fractions containing the mitochondrion-kinetoplast complex have been used as starting material to purify macromolecules whose origin can be ascribed undoubtedly to the complex. Schneider and co-workers (1994) demonstrated that *T. brucei* mitochondrial tRNAs are encoded in the nucleus and imported into the mitochondrion, where they are spliced. The authors modified tRNA genes by site-directed mutagenesis and found an accumulation of unspliced mutant tRNA in the mitochondrial fraction, thereby demonstrating that the tRNA had been imported. Using cell fractionation and immunofluorescence microscopy, Sullivan and co-workers (1994) localized a heat-shock protein of 60 kDa (hsp60) that acts

on mitochondrial protein folding. Unlike hsp70, which is associated with the kinetoplast, hsp60 is distributed all over the mitochondrial matrix. Recently, Heise and Opperdoes (2000) used a cell fractionation, isopycnic centrifugation and digitonin-titration protocol to purify the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase from procyclic *T. brucei*, and demonstrated that it is localized in the mitochondrion. This is the first description of an enzyme from the ergosterol synthesis pathway of *T. brucei*. The authors suggested that the mitochondrial localization of this enzyme, which is resident in the endoplasmic reticulum in mammalian cells (Lecureux & Wattenberg 1994), is due to the use of 2-carbon molecules from the metabolism of threonine and/or leucine as a source of acetyl groups for incorporation into sterols.

Isolation of the Golgi complex

The Golgi complex of trypanosomatids is formed by 4 to 10 stacked cisternae localized in the anterior region of the cell, close to the flagellar pocket. Biochemical studies have shown that in members of the Trypanosomatidae family the pathway of N-linked oligosaccharide processing of protein is similar to that found in other eukaryotic cells (Parodi et al. 1981, 1989, Parodi 1993). However, a novel series of unique O-linked N-acetylglucosamine-containing oligosaccharides have been found in sialoglycoproteins (Previato et al. 1994, 1995). It has been assumed, based on the results obtained with mammalian cells, that the Golgi complex of trypanosomatids is involved in the glycosylation process. Indeed, a few cytochemical studies in which thin sections of Lowicryl-embedded trypanosomatids were incubated with gold-labeled lectins showed labeling of the Golgi complex.

Two attempts have been made to purify the Golgi complex of trypanosomatids. The first one was carried out with African trypanosomes (Grab et al. 1984). Transmission electron microscopy showed that the fraction consisted predominantly of smooth surface vesicles and flattened cisternae rather than stacked Golgi cisternae. The second attempt was carried out on epimastigote forms of *T. cruzi* (Morgardo-Díaz et al. 2001). The parasites were suspended in a hypotonic solution containing protease inhibitors and then disrupted by controlled sonication followed by centrifugation at 95,000x g for 90 min in a discontinuous sucrose density gradient (1.2, 1 and 0.8 M sucrose). Transmission electron microscopy of a band recovered at a position corresponding to the 1-1.2 M sucrose interfaces was highly enriched in stacked cisternae and vesicles. The fraction was characterized biochemically as significantly enriched in galactosyl transferase, O-α-GlcNAc transferase and acid phosphatase (Morgado-Díaz et al. 2001). A protein of 58 kDa that has been shown to be involved in the association of the Golgi complex with microtubules in mammalian cells was found by immunofluorescence microscopy and Western blotting in the Golgi complex of *T. cruzi* (Morgado-Díaz et al. 2001).

Isolation of the glycosome (peroxisome)

Membrane-bound cytoplasmic structures that resemble those initially designated as microbodies and later on as peroxisomes in mammalian cells, have been described in trypanosomatids since the initial studies on their fine structure (Vickerman & Preston 1976, reviewed in De Souza 1984). The peroxisomes usually appear as spherical organelles with a diameter of about 0.7 μm and are randomly distributed throughout the cell. In some cells, as in *Leptomonas samueli*, they appear as elongated structures that can reach a length of 2.8 μm (Souto-Padrón & De Souza 1982). In some *Phytomonas* species, the peroxisomes may be packed arranged and even associated with cytoskeletal structures (Attias & De Souza 1985). In trypanosomatids that harbour an endosymbiont, the peroxisomes concentrate around the symbiont (Motta et al. 1997). Usually the glycosome contains a homogeneous and slightly dense matrix, although a crystalloid core can be seen in some protozoa such as *T. brucei* and *L. mexicana* (J Alexander, cited by Vickerman & Preston 1976).

Peroxisomes have been defined as organelles that are bounded by a single membrane and that contain catalase and H_2O_2-producing oxidases. Catalase is easily localized using the alkaline diaminobenzidine incubation medium and has been used as an enzyme marker, to identify an organelle as a peroxisome. The application of this approach in trypanosomatids led to the identification of two groups. One includes digenetic trypanosomatids such as *T. brucei, T. cruzi* and *Leishmania*, where no significant enzyme activity could be detected by either enzyme assay or cytochemistry. The other includes the monogenetic trypanosomatids such as *Crithidia, Leptomonas*, etc. where the enzyme is easily detected (Souto-Padrón & De Souza 1982).

Initial cell fractionation studies showed that the first seven enzymes of the glycolytic pathway are localized in the peroxisomes, rather than in the cytoplasm as in all other eukaryotes. The compartmentalization of glycolysis, first described in *Trypanosoma brucei* (Opperdoes & Borst 1977), was observed in all members of the Trypanosomatidae family (Cannata et al. 1982, Coombs et al. 1982, Taylor et al. 1979, Opperdoes et al. 1977) and the organelle was thus called a glycosome. Biochemical studies carried out on isolated fractions have shown that the glycosomes of trypanosomatids are involved in other metabolic pathways such as carbon dioxide fixation (Opperdoes & Cottem 1982), purine salvage and *de novo* pyrimidine biosynthesis (reviewed in Opperdoes 1987, Shih et al. 1998, Michels et al. 2000, Parsons et al. 2001). Opperdoes and co-workers obtained a highly purified subcellular fraction containing glycosomes from bloodstream-form trypomastigotes of *T. brucei*: grinding with silicon carbide disrupted the parasites and a glycosome-enriched fraction was recovered from a Percoll gradient. The organelle was further purified on a sucrose gradient, equilibrating at 1.23 g/cm^3 (Opperdoes et al. 1984). The authors achieved the separation of the glycosome membrane from its matrix by incubating the purified fraction on ice, at pH 11.5, in hypotonic buffer. Electron microscopy and biochemical analysis were used to evaluate the purity of the fraction and analyze the isolated organelle. Morphometric analysis in situ showed that glycosomes are round or ellipsoid compartments with a mean diameter of 0.27 μm, present in an average number of 230 per cell, representing 4.3% of the volume of the parasite. Biochemical data demonstrated the enrichment of several glyco-

lytic enzymes and the absence of mitochondrial contamination and DNA.

The same group applied the glycosome purification scheme to procyclics, the insect stage of *T. brucei* (Hart et al. 1984). Comparing the enzymatic activities of purified glycosomes from bloodstream and procyclic forms, the authors found lower glycolytic activities in the latter. On the other hand, they found adenyl kinase, malate dehydrogenase and phosphoenolpyruvate carboxykinase in procyclic glycosomes, enzymes that are also present in the mitochondrion (Opperdoes et al. 1981). The enzymes involved in glycerol metabolism had equivalent activities in both forms. A few years later, Aman and Wang (1986) compared glycosome fractions that had been isolated using three different gradients: organelles obtained from two sequential sucrose gradients were more purified than those obtained from gradients of Percoll or Nicodenz. In this paper, the authors compared enzymatic activities from purified glycosomes of procyclic and bloodstream form trypomastigotes, and found that glycolytic enzyme activities from procyclic forms were reduced, whereas activities of glycerol-modifying enzymes were maintained.

Purified glycosome fractions were permeabilized with toluene (McLaughlin 1985) or Triton X-100 after treatment with cross-linking agents (Aman et al. 1985), to test the activity of core enzymes. These experiments demonstrated that glycolytic enzymes are not bound to the membrane, but are closely arranged spatially.

Besides glycolysis, glucose can be metabolized via the pentose phosphate pathway. $NADP^+$-dependent glucose-6-phosphate dehydrogenase is an enzyme of this latter pathway that converts glucose-6-phosphate to ribose-5-phosphate to be used for nucleotide biosynthesis. Recently, it was found to be associated with glycosomes and with the cytoplasm of *T. brucei* procyclic promastigotes by testing enzyme latency with a digitonin titration experiment (Heise & Opperdoes 1999).

Isolation of the acidocalcisome

Acidocalcisomes are membrane-bounded organelles with an electron-dense content, acidic character and calcium storage capacity (reviewed in Docampo & Moreno 1999). The ultrastructural appearance of these organelles varies, as their inorganic content is not well preserved by chemical fixatives (Miranda et al. 2000), so that fixation results in an apparently empty vacuole with a peripheral dense deposit, previously known as volutin granules. The best preservation was obtained with high-pressure freezing followed by freeze substitution, revealing a very electron-dense aspect. Acidocalcisomes were detected in several protozoa: *T. brucei* (Vercesi et al. 1994), *T. cruzi* (Docampo et al. 1995), *L. amazonensis* (Lu et al. 1997), *Toxoplasma gondii* (Moreno & Zhong 1996) and *Plasmodium falciparum* (Marchesini et al. 2000). Biochemical data obtained from digitonin-permeabilized cells indicated the presence of a bafilomycin A-sensitive H^+-ATPase and a vanadate-sensitive Ca^{2+} pump in the acidocalcisome. Immunochemical approaches have detected some enzymes that could be responsible for these transport activities: a vacuolar H^+ ATPase (Moreno et al. 1998, Benchimol et al. 1998), a Ca^{2+} - H^+ translocating ATPase (Lu et al. 1998)

and a vacuolar-type H^+-pyrophosphatase. This last enzyme was considered similar to plant pyrophosphatases, as its activity was stimulated by K^+ and inhibited by Na^+ (Scott et al. 1998, Luo et al. 1999, Rodrigues et al. 1999a, 1999b, 2000).

The function of the acidocalcisome is still not completely clarified. Docampo and Moreno have recently (1999) suggested some possibilities, such as a storage role in calcium homeostasis and in energy metabolism, in view of the large accumulation of inorganic pyrophosphate (Urbina et al. 1999) Other possibilities include pH control and osmoregulation, and the presence of Na^+-H^+ exchange activity (Rodrigues et al. 1999b).

To properly assess the function of acidocalcisomes, it is necessary to study the membrane transport events listed above. A suitable preparation for such studies is a sub-cellular fraction containing well-preserved acidocalcisomes and no other cell membranes. The first attempt to isolate acidocalcisomes was made in 1997 (Scott et al. 1997): *T. cruzi* epimastigotes were treated with Triton WR-1339, to decrease the density of intracellular vacuoles, and lysed by grinding with silicon carbide. The organelles were then separated on a Percoll gradient. In a subsequent paper from Scott and Docampo (1998), an improvement was obtained by omitting the detergent treatment. The organelles were denser than glycosomes and were concentrated at the bottom of the gradient together with Percoll particles. This led to great loss of acidocalcisomes when Percoll was washed out. Similar methods were adopted to isolate acidocalcisomes from *L. donovani* (Rodrigues et al. 1999a) and *T. brucei* (Rodrigues et al. 1999b). Recently, a new protocol was established by Scott and Docampo (2000) using iodixinol, a density gradient solute that does not precipitate, allowing acidocalcisome isolation in good yield (Fig. 6). The presence of a H^+-pyrophosphatase was confirmed using isolated acidocalcisomes. However, different from plants, vacuolar H^+-ATPase was not found in the same compartment, although it has been detected in the same Ca^{2+}-containing compartment in permeabilized cells (Lu et al. 1998) and by immunocytochemistry (Benchimol et al. 1998). Isolated acidocalcisomes were probed for V-type H^+-ATPase using an antibody against a membrane subunit that should be detected even if peripheral subunits had been lost during isolation. These data leave the possibility that two populations of acidocalcisomes are present in epimastigotes: one less dense that bears both enzymes and another, denser form that was purified and lacks the ATPase (Scott & Docampo 2000).

Isolation of endocytic compartments

In trypanosomatids, few data concerning an endocytic pathway are available. Due to a subpellicular array of the parallel, closely arranged, stable microtubules, the only possible sites for the uptake of macromolecules are the flagellar pocket and the cytostome, in those forms where it is present. In *T. brucei*, coated vesicles bud from the flagellar pocket (Langreth & Balber 1975, Coppens et al. 1987, Webster 1989). Endocytosed proteins are subsequently found concentrated in a tubular network, which the authors named the endosome, and in round lysosome-

like organelles (Webster 1989, Webster & Fish 1989). Early endosomes, often closely associated with recycling compartments, were only recently characterized, using the small GTPases TbRab4 and TbRab5 as markers (Field et al. 1998). Although transferrin and its receptor are not recycled in *T. brucei*, as in other eukaryotic cells (Grab et al. 1992), the variant surface glycoprotein (VSG) is internalized and recycled. An early attempt to isolate endosomal compartments in *T. brucei* bloodstream forms was made in 1980, as part of a comprehensive fractionation scheme (Steiger et al. 1980). Hydrolase activities and latency were used as criteria to identify isolated fractions. In this way, α-mannosidase and acid proteinase, recovered in a gradient at a density of about 1.20 g/cm^3, were associated with lysosomes, while acid phosphatase, totally accessible in intact cells, was associated with the flagellar pocket. Some years later, coated vesicles were purified from *T. brucei* bloodstream trypomastigotes (Shapiro & Webster 1989). Parasites were lysed by freezing and thawing and coated vesicles were purified by subsequent sucrose and Percoll gradients. The analysis of the fraction showed that the vesicles contained VSG, which formed a coat on the inner face of the vesicle itself. They also contained IgG, IgM and serum albumin. Their cytoplasmic coat does not contain clathrin as a major component, as found in coated vesicles of mammalian cells. Recently, preparative free-flow electrophoresis was used to separate and analyze endocytic compartments from *T. brucei* that had taken up a fluid phase marker, horseradish peroxidase (Grab et al. 1998). At low temperatures, peroxidase was found in positively charged compartments, but did not co-localize with lysosomal proteases. When compared to isopycnic density gradient centrifugation, preparative free-flow electrophoresis proved to be more suitable to discriminate between only slightly different compartments.

In *T. cruzi* epimastigotes, the endocytic pathway is very polarized: proteins are captured mainly in the cytostome, in the cell's anterior region, close to the flagellar pocket (Porto-Carreiro et al. 2000) and stored in reservosomes, uncommon organelles always present at the posterior region of epimastigotes (De Souza et al. 1978a, Soares & De Souza 1988, 1991). We have obtained a highly purified subcellular fraction (Fig. 7) containing reservosomes from epimastigotes (Cunha-e-Silva et al. 2002). The protein profile of the organelle analyzed by SDS-PAGE revealed a wide range of protein bands, predominantly those corresponding to 85 kDa, a triplet of 67-57 kDa and a doublet of 24-23 kDa. Protease activity in substrate-containing gels, in the presence or absence of protease inhibitors, showed that cysteine proteinase is enriched and very active in the purified fraction. Enzymatic assays demonstrated the absence of pyrophosphatase, an acidocalcisome marker, and hexokinase, a glycosomal marker, although both enzymes were detected in other regions of the gradient. Western blot analysis using specific antibodies showed an enrichment of TcRab11, a GTPase present in the organelle in the intact epimastigote (Mendonça et al. 2000). Thin-layer chromatographic neutral lipid analysis of purified reservosomes demonstrated that the organelle stores large amounts of cholesterol and triacylglycerol. Phospholipid analysis indicated phosphatidylcholine and phosphatidylethanolamine as the major constituents of reservosome membranes.

CELL FRACTIONATION OF APICOMPLEXA

Fig. 8 shows a thin section of a trophozoite form of Apicomplexa represented by the haemogregarine *Cyrilia lignieresi*. The protozoan is surrounded by a complex
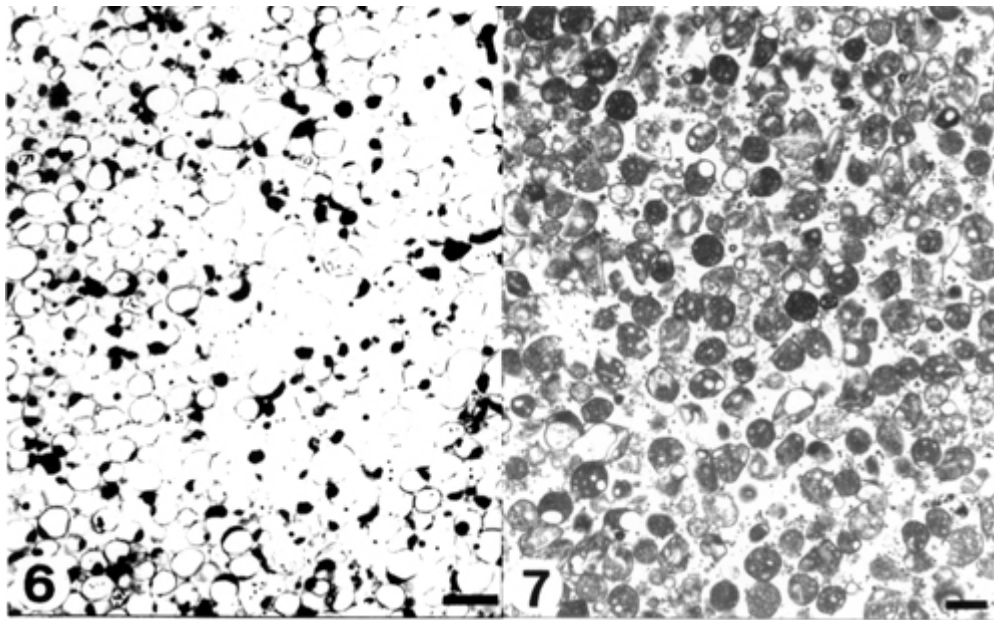

Fig. 6: acidocalcisome fraction isolated from *Trypanosoma brucei* procyclics (micrograph by Docampo and co-workers). Bar = 0.5 μm. Fig. 7: reservosomes enriched fraction from *Trypanosoma cruzi* (after Cunha-e-Silva et al. 2002) Bar = 1 μm

membrane system formed by an outer plasma membrane and two closely apposed unit membranes, which form the inner membrane complex, which is interrupted at some points of the cell body and at the very anterior region of the cell. A layer of microtubules is localized under the inner membrane complex. These microtubules originate from the inner ring of the conoid, a structure located at the apical pole, and project toward the posterior but do not reach the most posterior portion of the cell. The nucleus is located in the central portion. Four special organelles are found in the cytoplasm of the protozoan. The first is a group of small, cigar-shaped organelles that are restricted to the apical third of the protozoan body and known as micronemes. Their number varies according to the species and the developmental stages. In some they are hardly seen while in others they are so numerous that they are the most abundant organelle found in the cell (Fig. 8). The organelle is surrounded by a typical unit membrane and presents an electron-dense matrix due to its high protein content. Indeed, the organelle was intensely stained when the protozoa were subjected to the ethanolic phosphotungstic acid technique, which reveals basic proteins (De Souza & Souto-Padrón 1978). It has been shown that when the infective forms of Apicomplexan parasites touch the host cell surface a process of Ca^{2+} release is triggered with the subsequent discharge of the content of the micronemes at the junction between the parasite and the host cell (Carruthers et al. 1999). This material then mediates parasite attachment (Carruthers et al. 1999, Carruthers & Sibley 1999).

The second group of special organelles in Apicomplexa is formed by long, club-shaped organelles connected by thin necks to the extreme apical pole of the parasite and known as rhoptries (Fig. 8). At their basal portion the matrix of the organelle shows a spongy appearance, while the neck region is uniformly electron dense, making it difficult to distinguish from the micronemes. Their number varies according to the species. Several rhoptries can be seen in *T. gondii* whereas only two, often designated as the paired organelle, are found in *Plasmodium*. A typical unit membrane surrounds the organelle. Secretion of rhoptry proteins takes place immediately after adhesion of the parasites to the host cell surface. In the case of *T. gondii* kinetic studies showed that release of the proteins is completed in about 1 minute and that the proteins are internalized and will form part of the membrane lining the parasitophorous vacuole (Sam-Yellowe et al. 1988, Carruthers & Sibley 1997).

A third group comprises spherical organelles distributed throughout the cell rather than localized at the apical complex, with a mean diameter of about 0.7 µm, known as dense granules. Their matrix is uniformly electron dense due to the high concentration of protein. Kinetic studies have shown that secretion of the dense-granule content takes place after parasite invasion and localization within the parasitophorous vacuole, persisting for several minutes (Carruthers & Sibley 1997).

In contrast to secretion of micronemes and rhoptries, which takes place in the apical region, dense granule secretion occurs at the lateral regions of the protozoan. The secreted proteins associate with the membrane of the parasitophorous vacuole and with the parasite-derived intravacuolar membranous network.

The fourth organelle is surrounded by four membranes (reviewed in Gleeson 2000) and localized in the anterior region, next to the nucleus. Based on data showing that it contains a 35 kb DNA molecule similar to that found in the chloroplast genome, the term apicoplast has been suggested for this organelle (Kohler et al. 1997). The presence of the four unit membranes surrounding the apicoplast has been interpreted as evidence that it originated as a secondary endosymbiont derived by ingestion of a eukaryote that itself harbored a plastid, analogous to the plastids of chlorarachniophytes and cryptomonads.

Isolation of the membrane complex

Several attempts have been made to obtain isolated fractions containing the plasma membrane of protozoa of the Apicomplexa phylum. Ideally we should get a single fraction containing the plasma membrane, the inner membrane complex and the subpellicular microtubules. Subsequently, these components could be separated.

The most successful approach was that used by Dubremetz and co-workers (Foussard et al. 1991, Rabjeau et al. 1997) using tachyzoites of *T. gondii*. The parasites were disrupted by sonication and the homogenate subjected to differential centrifugation to pellet unbroken cells and the nuclear fraction. The 600 x g supernatant was then collected and sedimented at 30,000 x g for 30 min and the resultant pellet applied to discontinuous sucrose gradients. In a first attempt a 5-step sucrose gradient was used and a membrane enriched fraction was observed at the 0.25-1.0 M sucrose interface (Foussard et al. 1990). The purification was significantly improved by applying the post 600 x g supernatant to the bottom of a 4-step sucrose gradient (1.6, 1.4, 1.2 and 1.0 M), followed by centrifugation at 120,000 x g for 120 min. A layer at the 1.0-1.2 M sucrose interface was highly enriched in the pellicle containing the outer plasma membrane, the inner membrane complex and, in some cell fragments, even the subpellicular microtubules (Rabjeau et al. 1997). Two criteria were used to assess the purification: the morphology of the fraction as seen by transmission electron microscopy of thin sections (Fig. 9) and the enrichment in SAG 1, a well characterized plasma membrane protein detected by SDS-PAGE followed by Western blot. The 1.0-1.2 M interface fraction showed a 15-20-fold enrichment in SAG. Subsequently, the plasma membrane was separated from the whole pellicle by high salt-glycerol treatment followed by one cycle of freezing and thawing and sonication. The suspension was then centrifuged at 10,000 x g to sediment the undisrupted pellicles and the supernatant layered on top of sucrose gradient and centrifuged at 140,000 x g for 10 h. The centrifuge tube showed a pellet and a very faint low-density band close to the top of the gradient at buoyant density of 1.085 g/cm^3. This band contained well-conserved vesicles with diameters ranging from 20 to 100 nm. The nature of the vesicles was not determined.

Isolation of the rhoptries

The rhoptries of tachyzoites of *T. gondii* have been purified following rupture of the cells with a French press

operated at 28 kg/cm^3. Then, unbroken cells were pelleted by low-speed centrifugation and the homogenate was centrifuged at 20,000 x g for 15 min. The resulting pellet was then resuspended and adjusted to 30% Percoll and centrifuged at 50,000 x g for 25 min. Rhoptries were found in the bottom and then centrifuged again at 100,000 x g for 90 min to remove the Percoll particles yielding a highly enriched rhoptry fraction, as evaluated by transmission electron microscopy (Fig. 10) (Leriche & Dubremetz 1991). The fraction was, however, contaminated with dense granules and all attempts to separate the two organelles were unsuccessful. Only the lipids of the fractions were analyzed. Phospholipids were about two times lower while cholesterol was about five times higher in the fraction as compared to the total protozoan.

The rhoptries of *P. falciparum* were isolated starting with schizont-infected erythrocytes which were disrupted by nitrogen cavitation using a nitrogen-pressure chamber at 50 kg/cm^2 for 18 min followed by centrifugation at 8,000 x g for 10 min. The supernatant was collected and layered onto a linear 0.4-1.6 M sucrose gradient placed over a cushion of 2.2 M sucrose and centrifuged at 72,000 x g for 30 min. The layer above the gradient was removed and the gradient further centrifuged at 130,000 x g for 2.5 h after which fractions were collected and analyzed. Transmission electron microscopy of the fractions showed that the rhoptries were localized in fractions with densities of 1.155 and 1.166 g/cm^3. These two fractions were also enriched in the 110 and 140 kDa proteins previously identified as markers of the rhoptry (Holder et al. 1985, Sam-
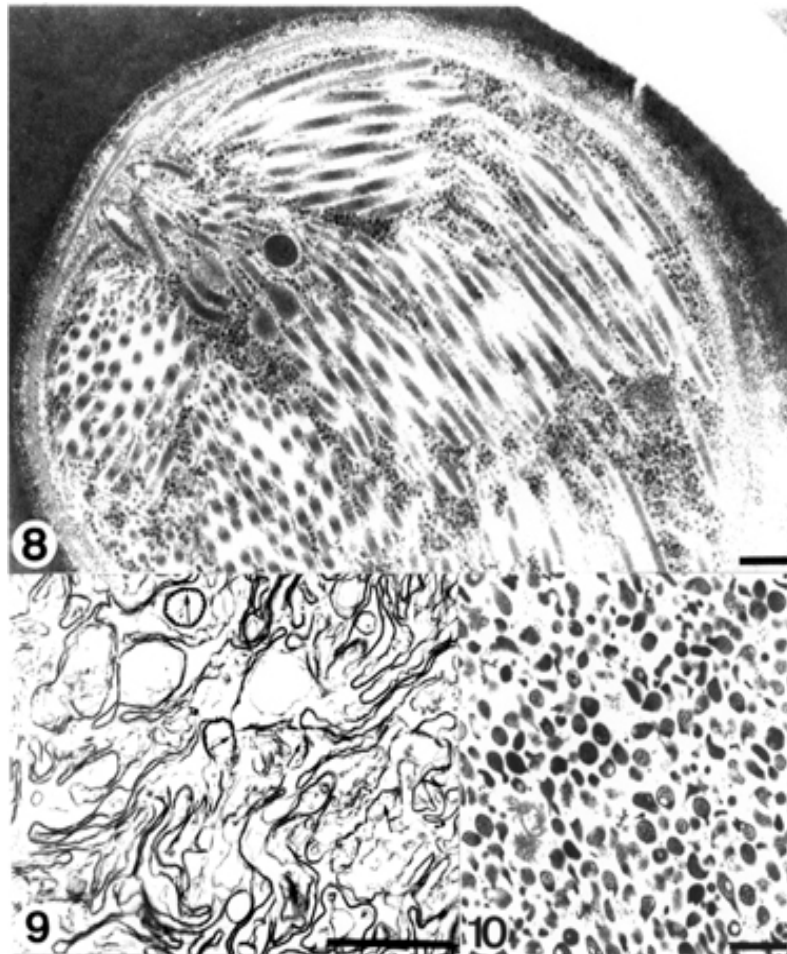

Fig. 8: anterior region of the haemogregarine *Cyrilia lignieresi*. Bar = 0.5 µm. Fig. 9: *Sarcocystis tenella* pellicular membrane fraction (after Dubremetz & Dissous 1980). Bar = 1 µm. Fig. 10: *Toxoplasma gondii* rhoptries fraction (after Leriche & Dubremetz 1991). Bar = 1 µm

Yellowe et al. 1988). SDS-PAGE of the rhoptry fraction showed the presence of a large number of proteins indicating the complexity of the organelle.

A rhoptry-containing fraction was also obtained from sporozoites of *Eimeria tenella* (Kawazoe et al. 1992). Oocysts were induced to sporulate and sporozoites purified by anion-exchange chromatography were disrupted by sonication and subjected to differential centrifugation. The 8,500 x g supernatant was centrifuged at 58,000 x g for 30 min in a sucrose gradient. The white layer at the top of each gradient was removed and re-centrifuged at 70,000 x g for 72 hours. The combined pellets from 1.0-1.6 M gradients were resuspended and layered onto a preformed gradient of 1.9-2.5 M sucrose and centrifuged at 62,000 x g for 2 h. A fraction collected at 2.3 M sucrose contained organelles with a diameter of 500-700 nm, which probably correspond to rhoptries. A large number of polypeptides were detected in this fraction by SDS-PAGE, including several of high molecular weights (118, 122, 124, 132, 180, and 220 kDa).

Isolation of the micronemes

In the attempts to obtain dense granules and rhoptries from *Sarcocystis* and *Eimeria*, as described above, a fraction containing structures similar to the micronemes was detected around 1.4 M sucrose (Fig. 11). SDS-PAGE showed that it had a relatively simple polypeptide content, with enrichment of bands at 26, 28, 31, 47, 130 kDa and one of > 200 kDa (Dubremetz & Dissous 1980, Dubremetz et al. 1989).

Isolation of the dense granules

Entzeroth and co-workers (1986) reported a procedure for the isolation of dense granules from zoites of *Sarcocystis muris*. The parasites were disrupted in a French pressure cell operated at 50 kg/cm^2 and the homogenate centrifuged at 500 x g for 10 min to eliminate intact or incompletely emptied cells. The supernatant was then submitted to a centrifugation at 12,000 x g for 10 min. The obtained pellet was re-suspended and adjusted to 1.4 M sucrose with 2.2 M sucrose, layered on a discontinuous sucrose gradient (2.0, 1.6, 1.4 and 0.25 M) and centrifuged for 1 h at 112,000 x g. The material detected at the 1.6-2 M interface was collected and sedimented for 45 min at 113,000 x g. The fraction obtained showed a large number of organelles, which by their size and shape correspond to the dense granules seen in intact cells (Fig. 12).

CELL FRACTIONATION OF TRICHOMONADIDAE

Fig. 13 shows a thin section of *Tritrichomonas foetus,* where the main structures and organelles observed in members of the Trichomonadidae family are depicted (reviewed in Honigberg 1978).

The protozoan is surrounded by a typical plasma membrane that envelops the cell body as well as the group of anterior flagella and the recurrent flagellum. The area of contact between the recurrent flagellum and the cell body is specialized and known as the undulating membrane. The cytoplasm of the protozoan shows a large number of free ribosomes, glycogen particles and vacuoles. This protozoan has a remarkable cytoskeleton, composed of
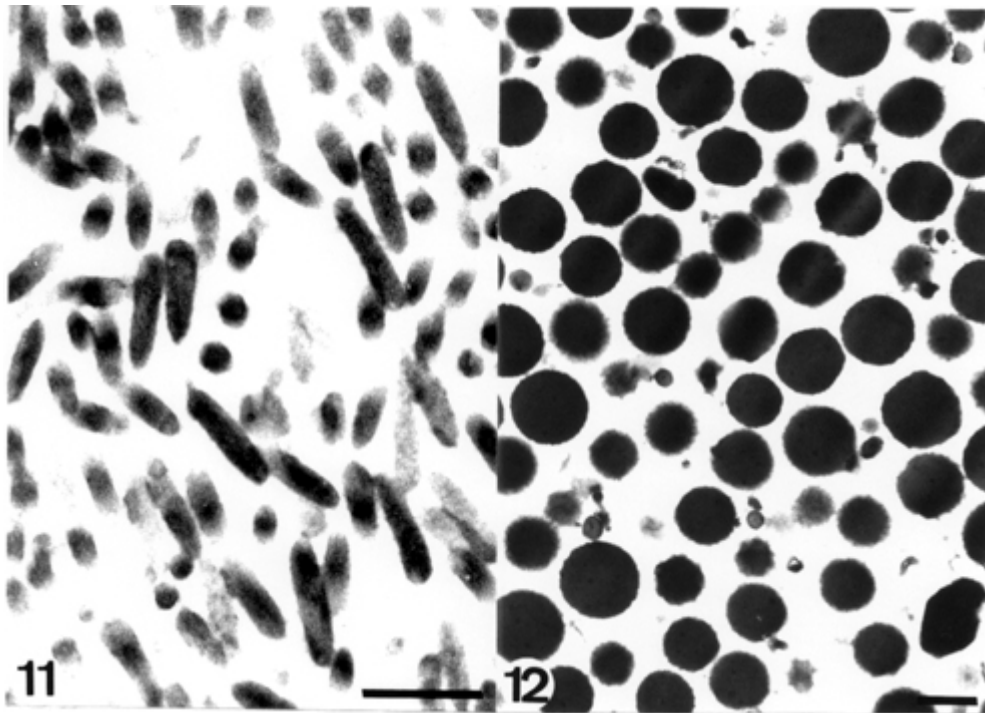

Fig. 11: *Sarcocystis tenella* microneme fraction (after Dubremetz & Dissous 1980). Bar = 0.5 μm. Fig. 12: *Sarcocystis muris* dense granules fraction (after Entzeroth et al. 1986). Bar = 1μm

several structures: (a) a sheet of microtubules that originate in the anterior end and project towards the posterior end, forming the pelta-axostylar complex; (b) a large striated structure known as the costa, also originating at the anterior end projecting towards the posterior region, usually subjacent to the region of contact of the recurrent flagellum with the cell body; and (c) a series of striated structures that originate close to the basal bodies and radiate in various directions, even establishing contact with the cis face of the Golgi complex. One characteristic feature of this group of protozoa is the presence of a large number of spherical organelles concentrated around the axostyle and the costa, known as hydrogenosomes. The hydrogenosome is a spherical or slightly elongated organelle of 0.5 to 1.0 mm in diameter, surrounded by two closely apposed membranes, which separate at certain points to form a peripheral vesicle (reviewed in Benchimol 1999) The hydrogenosome plays a fundamental role in the metabolism of the protozoan and is the site of production of ATP and molecular hydrogen (Muller 1993). The organelle has been the subject of intense investigation in recent years, especially by those interested in the process of evolution of cell organelles, since there are several similarities and differences between hydrogenosomes and mitochondria (Biagini et al. 1997, Muller 1997, Martin & Muller 1998, Hackstein et al. 1999).

Isolation of the plasma membrane

There is only one description of cell fractionation of trichomonads aimed at isolating the plasma membrane of *T. foetus* (Morgado-Díaz et al. 1996). The protozoa were collected and resuspended in buffer and disrupted with a Potter-type homogenizer. The homogenate was subjected to differential centrifugation and a microsomal fraction obtained as sediment after centrifugation at 80,000 x g. The resuspended pellet was applied to a discontinuous sucrose gradient (1.0, 1.3, 1.6 and 1.9 M) and centrifuged at 130,000 x g for 3 h. Several bands were collected and that localized at the sample-1.0 M sucrose interface was characterized as highly enriched in plasma membrane, as judged by electron microscopy and the assay of membrane marker enzymes. The fraction contained a population of small and large vesicles, some of which showed a multivesicular appearance. Enzyme assays showed an enrichment in the fraction of 5'-nucleotidase and Na^+-K^+-ATPase, two well characterized markers of the plasma membrane of eukaryotic cells and previously shown, using a cytochemical approach (Queiroz et al. 1991), to be localized in the plasma membrane of *T. foetus*.

Isolation of the Golgi complex

The Golgi complex of trichomonads is a large organelle consisting of 8-12 parallel cisternae. It has been isolated from *T. foetus* (Morgado-Díaz et al. 1996). The cells were disrupted with a Potter-type homogenizer and the homogenate centrifuged at 2,500 x g for 10 min to sediment unbroken cells, nuclei and other dense structures. The supernatant was mixed with 2.3 M sucrose to make a 1.4 M sucrose solution that was loaded into the bottom of a centrifuge tube, then overlaid in succession with 1.2, 1.0 and 0.8 M sucrose, and then centrifuged at 95,000 x g for 2.5 h. Two bands, corresponding to 0.8-1.0 and 1.0-1.2 M interfaces, designated as GF1 and GF2, respectively, were seen to contain cisternae and vesicles resembling the components of the Golgi complex. The GF2 fraction was significantly enriched in cisternae of the Golgi complex (Fig. 14) and highly enriched in galactosyltransferase, a well-known marker of the Golgi cisternae of eukaryotic cells. A preparation of GF1 and GF2 pooled together was sub-fractionated by sodium carbonate treatment and alkaline pH in order to separate the Golgi content and membrane fractions. About 15 protein bands were identified in the total Golgi purified fraction by SDS-PAGE (Morgado-Díaz & De Souza 1998).

Isolation of the hydrogenosome

The hydrogenosome has been the subject of excellent reviews published in the last years (Muller 1993, Biagini et al. 1997, Benchimol 1999). It appears as a spherical or slightly elongated structure with a mean diameter of 0.5 μm, reaching 2 μm in dividing organelles. The hydrogenosome is surrounded by two closely apposed unit membranes. Invaginations of the hydrogenosomal membrane forming inner compartments are occasionally seen. A characteristic feature of most of the hydrogenosomes is the presence of structures designated as peripheral vesicles. In some species only one or two vesicles are seen. In others, as is the case of *T. vaginalis*, several peripheral vesicles are found. The vesicle corresponds to about 8% of the volume of the hydrogenosome. Usually the vesicle is flat; however, its size and shape may vary significantly. When the cells are fixed in solutions containing calcium an electron dense product is seen within the vesicle. Cytochemical studies have shown that the peripheral vesicle is a site for the accumulation of calcium. The hydrogenosomal matrix usually appears as granular and homogeneous. The hydrogenosome does not contain nucleic acids. The hydrogenosomal proteins are synthesized in free cytoplasmic ribosomes and post-translationally inserted into the organelle via leader sequences which are absent from the mature protein and which are similar to mitochondrial pre-sequences.

The hydrogenosome compartmentalizes the fermentative metabolism of pyruvate, leading to the production of molecular hydrogen. Pyruvate generated by glycolysis or by conversion from malate is decarboxylated by pyruvate:ferredoxin oxidoreductase to the iron sulfur protein ferredoxin and subsequently to protons, forming molecular hydrogen. The enzyme hydrogenase participates in this step. Acetyl-CoA is converted to acetate with concomitant conversion of succinate into succinyl-CoA synthetase. The generation of acetyl CoA is coupled to ATP production via substrate-level phosphorylation. Interconversion of malate and pyruvate is catalyzed by a malate dehydrogenase which can utilize either NAD^+ or $NADP^+$. The hydrogenosome does not contain enzymes of the tricarboxylic acid cycle and does not carry out oxidative phosphorylation.

The hydrogenosome has been the subject of intense investigation from an evolutionary perspective. The absence of mitochondria in trichomonads and the fact that the hydrogenosome is surrounded by two membranes and participates in some metabolic pathways that take place

in the mitochondria of other cells strengthen this interest.

In view of the important role played by the hydrogenosome in the cell biochemistry and physiology of trichomonads, it has been the subject of intense investigation, including the isolation by cell fractionation techniques (Muller 1973). We present here one procedure, which yielded the purest fraction obtained for hydrogenosomes of *T. foetus* (Morgado-Díaz & De Souza 1997).

The cells were disrupted in a Potter-type homogenizer. The homogenization process was controlled by observation of the cells under a phase contrast microscope and stopped while some unbroken cells were still present. The resultant homogenate was subjected to differential centrifugation at 1,500 and 4,000 x g. The 4,000 x g pellet, which contained the hydrogenosomes, was layered on top of a gradient of 53, 45, 24 and 18% Percoll and then centrifuged at 36,000 x g for 30 min. A pure fraction containing well-preserved hydrogenosomes was recovered from the Percoll gradient. The purity of the fraction was assessed by electron microscopy (Fig. 15) with an enrichment of about 12 fold in malic decarboxylase, a marker enzyme of the hydrogenosome.

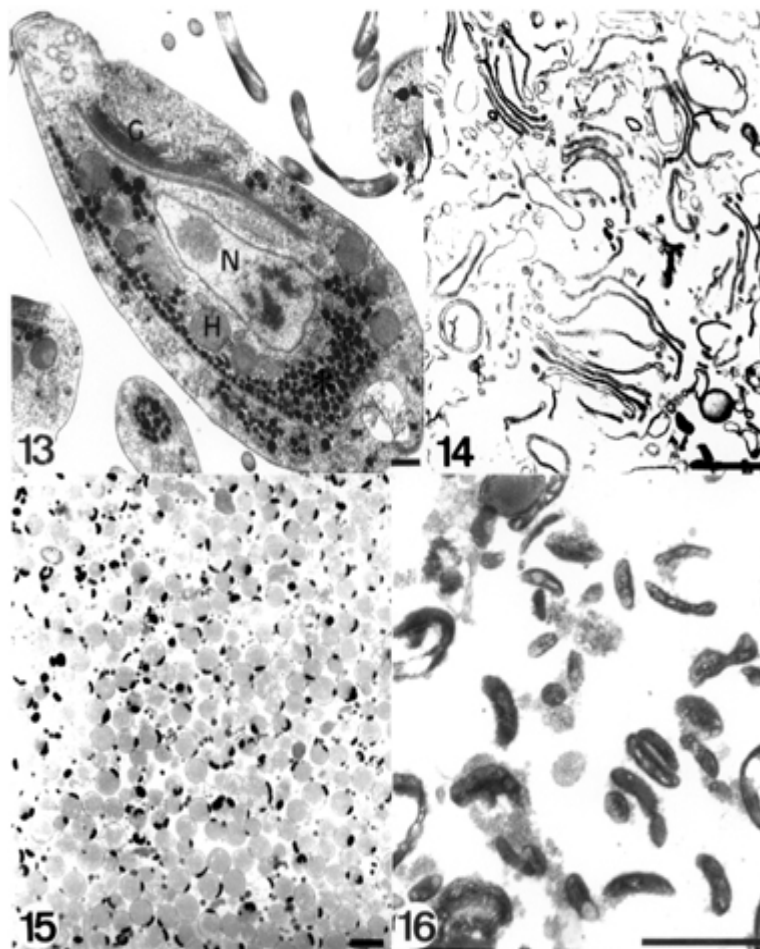As previously mentioned a flattened membrane-bound

Fig. 13: longitudinal section of *Tritrichomonas foetus* showing the nucleus (N), Golgi complex (G), hydrogenosomes (H) and glycogen particles (asterisk) (micrograph by M Benchimol). Bar = 0.5 μm. Fig. 14: purified Golgi complex fraction from *T. foetus* (after Morgado-Díaz et al. 1996). Bar = 0.5 μm. Fig. 15: purified hydrogenosome fraction from *T. foetus* (after Morgado-Díaz & De Souza 1997). Bar = 1 μm. Fig. 16: purified fraction of hydrogenosomal flat vesicles from *T. foetus* (after Morgado-Díaz & De Souza 1997). Bar = 0.5 μm

vesicle is observed at the periphery of the hydro-genosome. A pure hydrogenosomal fraction, as shown in Fig. 15, was resuspended in buffer containing 2% Triton X-100 and 50 mM 3-(N-morpholine) propanesulfonic acid (MOP-3)-NaOH, pH 7.0, and incubated on ice for 1 h. This treatment led to the complete solubilization of the membrane complex surrounding most of the organelle, but not the membrane lining the peripheral vesicle. In addition, components of the matrix remained attached to the vesicle. Further incubation of the fraction for 45 min at room temperature in the presence of proteinase K and subsequent centrifugation at 350,000 x g for 30 min gave a highly purified fraction containing only the peripheral vesicles (Fig. 16). This observation shows unequivocally that the peripheral vesicle is a special domain of the hydrogenosome. It is possible that it is involved in processes of regulation of cytoplasmic calcium concentration since cytochemical studies indicate that this cation accumulates in the peripheral vesicles (Benchimol et al. 1982a, Benchimol & De Souza 1983, De Souza & Benchimol 1988). The fractionation procedure was also monitored by analysis of the proteins using SDS-PAGE. Enrichment in about 12 polypeptide bands with molecular masses between 10 and 120 kDa was observed in the hydrogenosomal fraction. The peripheral vesicle showed few protein bands, with a major one of 45 kDa. Interestingly, this protein is an N-acetylglucosamine-containing glycoprotein, as demonstrated by Western blots probed with alkaline phosphatase-conjugated wheat germ agglutinin (WGA). This observation is in agreement with previous studies showing the presence of WGA-labeling sites only in the peripheral vesicle of hydrogenosomes found in intact cells (Benchimol et al. 1996). These results raise interesting questions concerning the mechanisms involved in the sorting of glycoproteins to a well-defined domain of the hydrogenosome, an organelle whose proteins are synthesized in free polyribosomes, released into the cytoplasm and subsequently translocated into the organelle (Lahti & Johnson 1991, Johnson et al. 1993). We cannot exclude the possibility of a close relationship between the peripheral vesicle and the endoplasmic reticulum.

Isolation of the costa

The costa is a cytoskeletal structure characteristic of members of the Trichomonadidae family, appearing as a striated root that originates near the basal bodies of the flagellum, and extends towards the posterior region of the protozoan. Some attempts have been made to isolate the costae. Taking advantage of the size of the costae of large trichomonads found in the gut of termites, and using micromanipulation it was possible to purify the structure from *T. gigantea* (Amos et al. 1979).

The first attempt made to isolate the costae of *T. foetus* yielded a cell fraction predominantly composed of carbohydrates (Lemoine et al. 1983, Sledge et al. 1978). However, cytochemical studies of intact cells showed that the costae were composed of proteins and did not contain carbohydrates (Benchimol et al. 1982b). The second cell fractionation approach yielded a highly enriched costa fraction from *T. foetus* (Monteiro-Leal et al. 1993). For this the protozoa were suspended in a medium containing 0.05% Lubrol PX detergent, washed in buffer and disrupted with a Dounce-type homogenizer. Cell disruption
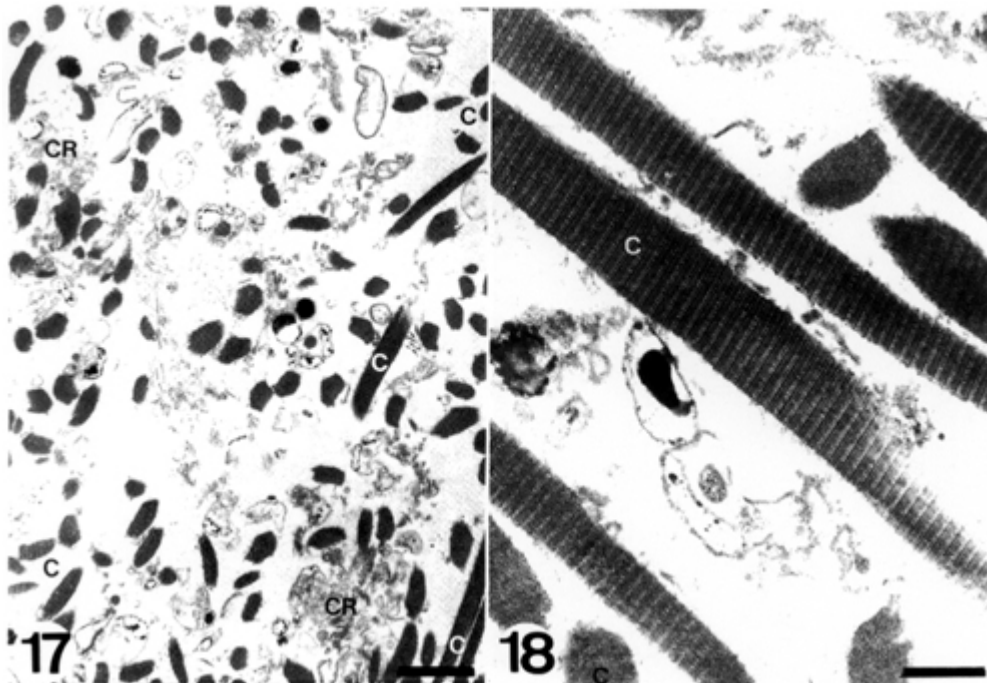

Fig. 17: costa enriched fraction from *Tritrichomonas foetus* (after Monteiro-Leal et al. 1993) containing costae (C) and chromatin (CR). Bar = 1 µm. Fig. 18: high magnification of costa fraction from *T. foetus* (After Monteiro-Leal et al. 1993). Bar = 0.5 µm

was monitored by light microscopy in an effort to obtain cell rupture without breaking the nuclear membrane. The homogenate was then vortexed and centrifuged for 15 min at 1000 x g over a 2 M sucrose cushion. The supernatant and the interface were pooled and layered on the top of a second cushion of 1.5 M sucrose and centrifuged for 15 min at 10,000 x g. The pellet was collected, layered on a four-step sucrose gradient (1, 1.5, 2 and 2.5 M sucrose from the top to the bottom) and centrifuged at 19,000 x g for 1 h. The material localized at the interface of 2/2.5 M was collected, resuspended in buffer and centrifuged at 10,000 x g for 10 min, yielding a highly enriched costa fraction (Figs 16, 17). SDS-PAGE analysis of the isolated fraction showed a group of polypeptide bands of 122, 115, 108, 93, 87 and 82 kDa. Further studies are necessary to determine the localization of each protein in the complex structural organization of the costae.

PERSPECTIVES

Cell fractionation is still the only approach that enables us to determine directly the chemical composition of an organelle, a cell structure or a region of the plasma membrane. As shown in this review, in some situations it is also possible to sub-fractionate an organelle into its components for further analysis. There also exists the possibility of isolating a given organelle in its different physiological states, which has been relatively little explored.

Studies carried out in mammalian cells have shown that it is possible to use cell fractionation techniques to isolate special domains of the plasma membrane. Such an approach should be used with parasitic protozoa, especially with those where there is evidence for the presence of specialized domains such as (a) the flagellar pocket of trypanosomatids, (b) the region of attachment of the flagellum to the cell body in trichomonads and trypanosomatids.

REFERENCES

Aman RA, Wang CC 1986. An improved purification of glycosomes from procyclic trypomastigotes of *Trypanosoma brucei*. *Mol Biochem Parasitol 21*: 211-220.

Aman RA, Kenyon GL, Wang CC 1985. Cross-linking of the enzymes in the glycosome of *Trypanosoma brucei*. *J Biol Chem 260*: 6966-6973.

Amos WB, Grimstone AV, Rothschild J, Allen RD 1979. Structure, protein composition and birefringence of the costa: a motile flagellar root fiber in the flagellate *Trichomonas*. *J Cell Sci 35*: 139-164.

Attias M, De Souza W 1985. Fine structure and cytochemistry of *Phytomonas davidi*. *J Submicrosc Cytol 17*: 205-212.

Bastin P, Matthews LR, Gull K 1996. The paraflagellar rod of kinetoplastida: solved and unsolved questions. *Parasitol Today 12*: 302-307.

Benchimol M 1999. The hydrogenosome. *Acta Microscop 8*: 1-22.

Benchimol M, De Souza W 1983. Fine structure and cytochemistry of the hydrogenosome of *Tritrichomonas foetus*. *J Protozool 30*: 422-425.

Benchimol M, Almeida JCA, De Souza W 1996. Further studies on the organization of the hydrogenosome in *Tritrichomonas foetus*. *Tiss Cell 28*: 287-299.

Benchimol M, De Souza W, Vanderheyden N, Zhong L, Lu H-G, Moreno S, Docampo R 1998. Functional expression of a vacuolar-type H$^+$-ATPase in the plasma membrane and intracellular vacuoles of *Trypanosoma cruzi*. *Biochem J 332*: 695-702.

Benchimol M, Elias CA, De Souza W 1982a. *Tritrichomonas foetus*: ultrastructural localization of calcium in the plasma membrane and the hydrogenosomes. *Exp Parasitol 54*: 277-284.

Benchimol M, Elias CA, De Souza W 1982b. *Tritrichomonas foetus*: ultrastructural localization of basic proteins and carbohydrates. *Exp Parasitol 54*: 135-144.

Biagini GA, Finlay BJ, Lloyd D 1997. Evolution of the hydrogenosome. *FEMS Microbiol Lett 155*: 133-140.

Braly P, Simpson L, Kretzer F 1974. Isolation of kinetoplast-mitochondrial complexes from *Leishmania tarentolae*. *J Protozool 21*: 782-790.

Cannata JJ, Valle E, Docampo R, Cazzulo JJ 1982. Subcellular localization of phosphoenolpyruvate carboxykinase in the trypanosomatids *Trypanosoma cruzi* and *Crithidia fasciculata*. *Mol Biochem Parasitol 6*: 151-160.

Carruthers VB, Sibley LD 1997. Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell Biol 73*: 114-123.

Carruthers VB, Sibley LD 1999. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol 31*: 421-428.

Carruthers VB, Moreno SN, Sibley LD 1999. Ethanol and acetaldehyde elevate intracellular [Ca^{2+}] and stimulate microneme discharge in *Toxoplasma gondii*. *Biochem J 342*: 379-386.

Claude A 1975. The coming of age of the cell. *Science 189*: 433-435.

Coombs GH, Craft JA, Hart DT 1982. A comparative study of *Leishmania mexicana* amastigotes and promastigotes. Enzyme activities and subcellular locations. *Mol Biochem Parasitol 5*: 199-211.

Coppens I, Opperdoes F, Courtoy PJ, Baldhuin P 1987. Receptor mediated endocytosis in the bloodstream form of *Trypanosoma brucei*. *J Protozool 34*: 465-473.

Cunha-e-Silva NL, Atella GC, Porto-Carreiro IA, Morgado-Diaz JA, Pereira MG, De Souza W 2002. Isolation and characterization of a reservosome fraction from *Trypanosoma cruzi*. *FEMS Microbiol Lett 214*: 7-12.

Cunha-e-Silva NL, De Souza W, Hasson-Voloch A 1984. Isolation of the flagellum and characterization of the paraxial structure of *Herpetomonas megaseliae*. *J Submicroscop Cytol 16*: 705-713.

Cunha-e-Silva NL, Hassón-Voloch A, De Souza W 1989. Isolation and characterization of a highly purified flagellar membrane fraction from trypanosomatids. *Mol Biochem Parasitol 37*: 129-136.

De Duve C 1967. Enzyme cytology. In Roodyn DB, Academic Press, New York.

De Duve C 1971. Tissue fractionation. Past and present. *J Cell Biol 50*: 20d-55d.

De Duve C 1975. Exploring cells with a centrifuge. *Science 189*: 186-194.

De Duve C, Beaufay, HA 1981. A short history of tissue fractionation. *J Cell Biol 91*: 293s-299s.

De Souza, W 1984. Cell biology of *Trypanosoma cruzi*. *Int Rev Cytol 86*: 197-282.

De Souza W 1989. Components of the cell surface of trypanosomatids. *Prog Protistol 3*: 87-184.

De Souza W 1995. Structural organization of the cell surface of pathogenic protozoa. *Micron 26*: 405-430.

De Souza W, Benchimol M 1988. Electron spectroscopic imaging of calcium in the hydrogenosomes of *Tritrichomonas foetus*. *J Submicroscop Cytol Pathol 20*: 619-621.

De Souza W, Souto-Padrón T 1978. Ultrastructural localization of basic proteins on the conoid, rhoptries and micronemes of *Toxoplasma gondii*. *Z Parasitenkd 56*: 123-129.

De Souza W, Souto-Padrón T 1980. The paraxial structure of the flagellum of Trypanosomatidae. *J Parasitol 66*: 229-235.

De Souza W, Chavez B, Martinez-Palomo A 1979. Freeze-fracture study of the cell membrane of *Herpetomonas samuelpessoai*. *J Parasitol 65*: 109-116.

De Souza W, De Carvalho TU, Benchimol M, Chiari E 1978a. *Trypanosoma cruzi*: ultrastructural, cytochemical and freeze-fracture studies of protein uptake. *Exp Parasitol 45*: 101-115.

De Souza W, Martinez Palomo A, Gonzales-Robles A 1978b. Cell surface of *Trypanosoma cruzi*: cytochemistry and freeze fracture. *J Cell Sci 33*: 285-294.

Docampo R, Moreno SNJ 1999. Acidocalcisome: A novel Ca^{2+} storage compartment in trypanosomatids and Apicomplexan parasites. *Parasitol Today 15*: 443-448.

Docampo R, Scott DA, Vercesi AE, Moreno SNJ 1995. Intracellular Ca^{2+} storage in acidocalcisomes of *Trypanosoma cruzi*. *Biochem J 310*: 1005-1012.

Dubremetz JF, Dissous C 1980. Characteristic proteins of micronemes and dense granules from *Sarcocystis tenella* zoites (Protozoa, Coccidia). *Mol Biochem Parasitol 1*: 279-289.

Dubremetz JF, Ferreira E, Dissous C 1989. Isolation and partial characterization of rhoptries and micronemes from *Eimeria nieschulzi* zoites (Sporozoa, Coccidia). *Parasitol Res 75*: 449-454.

Dwyer DM. 1980. Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes. *J Protozool 27*: 176-182.

Dwyer DM, D'Alessandro PA. 1980. Isolation and characterization of the pellicular membranes from *Trypanosoma lewisi* bloodstream forms. *J Parasitol 66*: 377-389.

Edwards C, Lloyd D 1977. Subcellular fractionation by differential and zonal centrifugation of the trypanosomatid *Crithidia fasciculata*. *J Gen Microbiol 100*: 339-346.

Entzeroth R, Dubremetz JF, Hodick D, Ferreira E 1986. Immunoelectron-microscopic demonstration of the exocytosis of dense granule contents into the secondary parasitophorous vacuole of *Sarcocystis muris* (Protozoa, Apicomplexa). *Eur J Cell Biol 41*: 182-188.

Farina M, Attias M, Souto-Padrón T, De Souza W 1986. Further studies on the organization of the paraxial rod of trypanosomatids. *J Protozool 33*: 552-557.

Field H, Farjah M, Pal A, Gull K, Field M 1998. Complexity of trypanosomatid endocytosis pathways revealed by Rab4 and Rab5 isoforms in *Trypanosoma brucei*. *J Biol Chem 273*: 32102-32110.

Foussard F, Gallois Y, Trouchin G, Robert R, Mauras G 1990. Isolation of the pellicle of *Toxoplasma gondii* (Protozoa, Coccidia): Characterization by electron microscopy and protein composition. *Parasitol Res 76*: 563-565.

Foussard F, Lerici MA, Dubremetz JF 1991. Characterization of the lipid content of *Toxoplasma gondii*. *Parasitology 102*: 367-370

Fouts DL, Stryker GA, Gorski KS, Miller MJ, Nguyen TV, Wrightsman RA, Manning JE 1998. Evidence for four distinct major protein components in the paraflagellar rod of *Trypanosoma cruzi*. *J Biol Chem 273*: 21846-21855.

Franco da Silveira J, Colli W 1981. Chemical composition of the plasma membrane from epimastigote forms of *Trypanosoma cruzi*. *Biochim Biophys Acta 644*: 341-350.

Franco da Silveira J, Abrahamson PA, Colli W 1979. Plasma membrane vesicles isolated from epimastigote forms of *Trypanosoma cruzi*. *Biochim Biophys Acta 550*: 222-232.

Freymuller E, Camargo EP 1981. Ultrastructural differences between species of trypanosomatids with and without endosymbionts. *J Protozool 28*: 175-182.

Fuge H 1969. Electron microscopic studies of the intra-flagellar structures of trypanosomes. *J Protozool 16*: 160-166.

Gleeson MT 2000. The plastid in Apicomplexa: what use is it? *Int J Parasitol 30*: 1053-1070.

Grab DJ, Ito S, Kara UAK, Rovis L 1984. Glycosyltransferase activities in Golgi complex and endoplasmic reticulum fractions isolated from African trypanosomes. *J Cell Biol 99*: 569-577.

Grab DJ, Webster P, Lonsdale-Eccles JD 1998. Analysis of trypanosomal endocytic organelles using preparative free-flow electrophoresis. *Electrophoresis 19*: 1162-1170.

Grab DJ, Wells CW, Shaw MK, Webster P, Russo DC 1992. Endocytosed transferrin in African trypanosomes is delivered to lysosomes and may not be recycled. *Eur J Cell Biol 59*: 398-404.

Hackstein JHP, Akhmanova A, Bosma B, Harjangi HR, Voncken FGJ 1999. Hydrogenosomes: eukaryotic adaptations to anaerobic environments. *Trends Microbiol 7*: 441-447.

Hart DT, Misset O, Edwards SW, Opperdoes FR 1984. A comparison of the glycosomes (microbodies) isolated from *Trypanosoma brucei* bloodstream form and cultured procyclic trypomastigotes. *Mol Biochem Parasitol 12*: 25-35.

Heise N, Opperdoes FR 2000. Localization of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the mitochondrial matrix of *Trypanosoma brucei* procyclics. *Z Naturforsch 55*: 473-477.

Heise N, Opperdoes FR 1999. Purification, localization and characterization of glucose-6-phosphate dehydrogenase of *Trypanosoma brucei*. *Mol Biochem Parasitol 99*: 21-32.

Hill GC, Brown CA, Clark MV 1968. Structure and function of mitochondria in C*rithidia fasciculata*. *J Protozool 15*: 102-109.

Holder AA, Freeman RR, Uni S, Aikawa M 1985. Isolation of a *Plasmodium falciparum* rhoptry protein. *Mol Biochem Parasitol 14*: 293-303.

Honigberg BM 1978. Trichomonads of veterinary importance. In*: JP Kreir, Parasitic Protozoa*, Academic Press, New York, p. 163-273.

Hunt RC, Ellar DJ 1974. Isolation of the plasma membrane of a trypanosomatid flagellate: General characterization and lipid composition. *Biochim Biophys Acta 339*: 173-189.

Imboden M, Müller N, Hemphill A, Mattioli R, Seebeck T 1995. Repetitive proteins from the flagellar cytoskeleton of African trypanosomes are diagnostically useful antigens. *Parasitology 110*: 249-258.

Johnson PJ, Lahti CJ, Bradley PJ 1993. Biogenesis of the hydrogenosomes in the anaerobic protist *Trichomonas vaginalis*. *J Parasitol 79*: 664-670.

Kawazoe U, Tomley FM, Frazier JA 1992. Fractionation and antigenic characterization of organelles of *Eimeria tenella*

sporozoites. *Parasitology 104*: 1-9.

Kohler S, Delwiche CF, Denny PW, Tilney LG, Webster P, Wilson RJ, Palmer JD, Roos, DS 1997. A plastid of probable green algal origin in Apicomplexan parasites. *Science 275*: 1485-1489.

Lahti CJ, Johnson PJ 1991. *Trichomonas vaginalis* hydrogenosomal proteins are synthesized on free polyribosomes and may undergo processing upon maturation. *Mol Biochem Parasitol 46*: 307-310.

Langreth SG, Balber AE 1975. Protein uptake and digestion in blood stream and culture forms of *Trypanosoma brucei. J Protozool 22:* 40-53.

Lecureux LW, Wattenberg BW 1994. The regulated degradation of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase reporter construct occurs in the endoplasmic reticulum. *J Cell Sci 107*: 2635-2642.

Lemoine LA, Hart LT, Larson AD, McCorkle-Shirley S 1983. Costa as a glycogen store and probable site of energy generation in *Tritrichomonas foetus. Am J Vet Res 44*: 713-714.

Leriche MA, Dubremetz JF 1991. Characterization of the protein contents of rhoptries and dense granules of *Toxoplasma gondii* tachyzoites by subcellular fractionation and monoclonal antibodies. *Mol Biochem Parasitol 45*: 249-260.

Linder JC, Staehelin CA. 1977. Plasma membrane specializations in a trypanosomatid flagellate. *J Ultrastruct Res 60*: 246-262.

Lindmark DG, Muller M 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. *J Biol Chem 248*: 7724-7728.

Lu H-G, Zhong L, Chang KP, Docampo R 1997. Intracellular Ca^{2+} pool content and signaling, and expression of a calcium pump are linked to virulence in *Leishmania mexicana amazonensis. J Biol Chem 272*: 9464-9473

Lu H-G, Zhong L, De Souza W, Benchimol M, Moreno SJN, Docampo R 1998. Ca^{2+} content and expression of an acidocalcisomal calcium pump are elevated in intracellular forms of *Trypanosoma cruzi. Mol Cell Biol 18*: 2309-2323.

Luo SH, Marchesini N, Moreno SNJ, Docampo R 1999. A plant-like vacuolar H^+-pyrophosphatase in *Plasmodium falciparum. FEBS Lett 460*: 217-220.

Mancini DE, Strickler JE, Patton CL 1982. Identification and partial characterization of the plasma membrane polypeptides of *Trypanosoma brucei. Biochim Biophys Acta 688*: 399-410.

Marchesini N, Luo S, Rodrigues CO, Moreno SNJ, Docampo R 2000. Acidocalcisomes and a vacuolar H^+-pyrophosphatase in malarial parasites. *Biochem J 347*: 243-253.

Martin W, Muller M 1998. The hydrogen hypothesis for the first eukaryote. *Nature 392*: 37-41.

McLaughlin J 1985. The presence of alpha-glycerophosphate dehydrogenase (NAD^+-linked) and adenyl kinase as core and integral membrane enzymes respectively in the glycosomes of *Trypanosoma rhodesiense. Mol Biochem Parasitol 14*: 219-230.

Mendonça SM, Da Silva JLN, Cunha-e-Silva NL, De Souza W, Lopes UG 2000. Characterization of a Rab11 homologue in *Trypanosoma cruzi. Gene 243*: 179-185.

Meyer H, De Souza W 1976. Electron microscopic study of *Trypanosoma cruzi* periplast in tissue cultures. I. Number and arrangement of the peripheral microtubules in the various forms of the parasite's life cycle. *J Protozool 23*: 385-390.

Michels PAM, Hannaert V, Bringaud F 2000. Metabolic aspects of glycosomes in Trypanosomatidae – New data and views. *Parasitol Today 16*: 482-489.

Miller M J, Wrightsman RA, Manning, JE 1996. *Trypanosoma cruzi*: protective immunity in mice immunized with paraflagellar rod proteins is associated with a T-helper type 1 response. *Exp Parasitol 84*: 156-167.

Miller MJ, Wrightsman RA, Stryker GA, Manning JE 1997. Protection of mice against *Trypanosoma cruzi* by immunization with paraflagellar rod proteins requires T cell, but not B cell, function. *J Immunol 158*: 5330-5337.

Miranda K, Benchimol M, Docampo R, De Souza W 2000. The fine structure of acidocalcisomes in *Trypanosoma cruzi. Parasitol Res 86*: 373-384.

Monteiro-Leal LH, Cunha-e-Silva NL, Benchimol M, De Souza W 1993. Isolation and biochemical characterization of the costa of *Tritrichomonas foetus. Eur J Cell Biol 60*: 235-242.

Moreira-Leite FF, De Souza W, Cunha-e-Silva NL 1999. Purification of the paraflagellar rod of the trypanosomatid *Herpetomonas megaseliae* and identification of its minor components. *Mol Biochem Parasitol 104*: 131-140.

Moreno SNJ, Zhong L 1996. Acidocalcisomes in *Toxoplasma gondii* tachyzoites. *Biochem J 813*: 655-659.

Moreno SNJ, Zhong L, De Souza W, Benchimol M 1998. Vacuolar-type H^+-ATPase regulates cytoplasmic pH in *Toxoplasma gondii. Biochem J 330*: 853-860.

Morgado-Díaz JA, De Souza W 1997. Purification and biochemical characterization of the hydrogenosomes of the flagellate protozoan *Tritrichomonas foetus. Eur J Cell Biol 74*: 85-91.

Morgado-Díaz J, De Souza W 1998. Biochemical characterization of the Golgi complex proteins of *Tritrichomonas foetus. Parasitol Res 84*: 760-762.

Morgado-Díaz J, Monteiro-Leal LH, De Souza W 1996a. Isolation and biochemical characterization of the plasma membrane of *Tritrichomonas foetus. Biocell 20*: 21-31.

Morgado-Díaz J, Monteiro-Leal LH, De Souza W 1996b. *Tritrichomonas foetus*: isolation and characterization of the Golgi complex. *Exp Parasitol 83*: 174-183.

Morgado-Díaz J, Nakamura CV, Agrillos OA, Dias WB, Previato JO, Mendonça-Previato LA, De Souza W 2001. Isolation and characterization of the Golgi complex of the protozoan *Trypanosoma cruzi. Parasitology 123*: 33-43.

Motta MC, Soares MJ, Attias M, Morgado J, Lemos AP, Saad-Nehme J, Meyer-Fernandes JR, De Souza W 1997. Ultrastructural and biochemical analysis of the relationship of *Crithidia deanei* with its endosymbiont. *Eur J Cell Biol 72*: 370-377.

Motta MCM, Sole-Cava AM, Faria-e-Silva PM, Fiorini JE, Soares MJ, De Souza W 1991. Morphological and biochemical characterization of the trypanosomatids *Crithidia desouzai* and *Herpetomonas anglusteri. Can J Zool 69*: 571-577.

Muller M 1973. Biochemical cytology of trichomonad flagellates. Subcellular localization of hydrolases, dehydrogenases and catalase in *Tritrichomonas foetus. J Cell Biol 57*: 453-474

Muller M 1997. Evolutionary origins of the trichomonads hydrogenosomes. *Parasitol Today 5*: 166-167.

Muller M 1993. The hydrogenosome. *J Gen Microbiol 139*: 2879-2889.

Müller N, Hemphill A, Imboden M, Duvallet G, Dwinger RH, Seebeck T 1992. Identification and characterization of two repetitive non-variable antigens from African trypanosomes which are recognized early during the infection. *Parasitology 104*: 111-120.

Nichols JM, Cross GA 1977. Isolation of mitochondria and mitochondrial RNA from *Crithidia fasciculata. J Gen Microbiol 99*: 291-300.

Opperdoes FR 1981. A rapid method for the isolation of intact

glycosomes in *Trypanosoma brucei*. *Mol Biochem Parasitol 3*: 181-186.

Opperdoes FR 1987. Compartmentalization of carbohydrate metabolism in trypanosomes. *Ann Rev Microbiol 41*: 127-151.

Opperdoes FR, Borst P 1977. Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: The glycosome. *FEBS Lett 80*: 360-364.

Opperdoes FR, Cottem D 1982. Involvement of the glycosome of *Trypanosoma brucei* in carbon dioxide fixation. *FEBS Lett 143*: 60-64.

Opperdoes FR, Baudhuin P, Coppens I, De Roe C, Edwards SW, Weijers P, Misset O 1984. Purification, morphometric analysis, and characterization of the glycosomes (microbodies) of the protozoan hemoflagellate *Trypanosoma brucei*. *J Cell Biol 98*: 1178-1184.

Opperdoes FR, Borst P, Bakker S, Leene, W 1977. Localization of glycerol-3-phosphate oxidase in the mitochondrion and NAD$^+$-linked glycerol-3-phosphate dehydrogenase in the microbodies of the bloodstream form of *Trypanosoma brucei*. *Eur J Biochem 76*: 29-39.

Opperdoes FR, Markos A, Steiger RF 1981. Localization of malate dehydrogenase, adenyl kinase and glycolytic enzymes in glycosomes and the threonine pathway in the mitochondrion of cultured procyclic trypomastigotes of *Trypanosoma brucei*. *Mol Biochem Parasitol 4*: 291-309.

Palade G 1975. Intracellular aspects of the process of protein synthesis. *Science 189*: 347-358.

Parodi AJ 1993. Glycosylation in trypanosomatid protozoa. *Glycobiology 3*: 196-199.

Parodi AJ, Martin-Barrients J, Engel JC 1989. Glycoprotein assembly in *Leishmania mexicana*. *Biochem Biophys Res Comm 118*: 1-8.

Parodi AJ, Quesada-Allue, LA, Cazzulo JJ 1981. Pathway of protein glycosylation in the trypanosomatid *Crithidia fasciculata*. *Proc Nat Acad Sci USA 78*: 6201-6205.

Parsons M, Furuya T, Pal S, Kessler P 2001. Biogenesis and function of peroxisomes and glycosomes. *Mol Biochem Parasitol 115*: 19-28.

Pereira NM, De Souza W, Machado RD, De Castro FT 1977. Isolation and properties of the flagella of trypanosomatids. *J Protozool 24*: 511-514.

Pereira NM, Timm SL, Da Costa SCG, Rebelo MA, De Souza W 1978. *Trypanosoma cruzi*: Isolation and characterization of membrane and flagellar fractions. *Exp Parasitol 46*: 561-566.

Piras MM, De Rodriguez OO, Piras R 1981. *Trypanosoma cruzi*: antigenic composition of axonemes and flagellar membranes of epimastigotes cultured *in vitro*. *Exp Parasitol 51*: 59-73.

Porto-Carreiro I, Attias M, Miranda K, De Souza W, Cunha-e-Silva NL 2000. *Trypanosoma cruzi* epimastigotes endocytic pathway: cargo enters the cytostome and passes through an early endosomal network before reservosome storage. *Eur J Cell Biol 79*: 858-869.

Previato JO, Jones C, Gonçalves LPB, Wait R, Travassos LR, Mendonça-Previato, L 1994. O-glycosidically linked N-acetyl glucosamine-bound oligosaccharide from glycoproteins of *Trypanosoma cruzi*. *Biochem J 301*: 151-159.

Previato JO, Jones C, Xavier MT, Wait R, Travassos LR, Parodri AJ, Mendonça-Previato, L 1995. Structural characterization of the major glycosylphosphatidylinositol membrane anchored glycoprotein of epimastigote forms of *Trypanosoma cruzi* Y-strain. *J Biol Chem 270*: 7241-7250.

Queiroz RCB, Silva Santos LM, Benchimol M, De Souza W 1991. Cytochemical localization of enzyme markers in *Tritrichomonas foetus*. *Parasitol Res 77*: 561-566.

Rabjeau A, Foussard F, Mauras G, Dubremetz JF 1997. Enrichment and biochemical characterization *of Toxoplasma gondii* tachyzoite plasmalemma. *Parasitology 114*: 421-426.

Rodrigues CO, Scott DA, Bailey B, De Souza W, Benchimol M, Urbina J, Oldfield E, Moreno SNJ 2000. Vacuolar proton pyrophosphatase activity and pyrophosphate in *Toxoplasma gondii* as possible chemotherapeutic targets. *Biochem J 349* :737-745.

Rodrigues CO, Scott DA, Docampo R 1999a. Presence of a vacuolar H$^+$-pyrophosphatase in promastigotes of *Leishmania donovani* and its localization to a different compartment from the vacuolar H$^+$-ATPase. *Biochem J 340*: 759-766.

Rodrigues CO, Scott DA, Docampo R 1999b. Characterization of a vacuolar pyrophosphatase in *Trypanosoma brucei* and its localization to acidocalcisomes. *Mol Cell Biol 19*: 7712-7723.

Rovis L, Baekkeskov S 1980. Sub-cellular fractionation of *Trypanosoma brucei*. Isolation and characterization of plasma membrane. *Parasitology 80*: 507-524.

Ruiz AM, Esteva M, Meckert PC, Laguens RP, Segura EL 1985. Protective immunity and pathology induced by inoculation of mice with different subcellular fractions of *Trypanosoma cruzi*. *Acta Trop 42*: 299-309.

Ruiz AM, Esteva M, Riarte A, Subías E, Segura EL 1986. Immunoprotection of mice against *Trypanosoma cruzi* with a lyophilized flagellar fraction of the parasite plus adjuvant. *Immunol Lett 12*: 1-4.

Russel DG, Newsam RJ, Palmer GCN, Gull K 1983. Structural and biochemical characterisation of the paraflagellar rod of *Crithidia fasciculata*. *Eur J Cell Biol 30*: 137-143.

Sam-Yellowe TY, Shio H, Perkins ME 1988. Secretion of *Plasmodium falciparum* rhoptry proteins into the plasma membrane of host erythrocytes. *J Cell Biol 106*: 1507-1513.

Schneider A, Lutz HU, Marug R, Gehr P, Seebeck T 1988. Spectrin-like proteins in the paraflagellar rod of *Trypanosoma brucei*. *J Cell Sci 90*: 307-315.

Schneider A, Martin J, Agabian N 1994. A nuclear encoded tRNA of *Trypanosoma brucei* is imported into mitochondria. *Mol Cell Biol 14*: 2317-2322.

Scott DA, Docampo R 1998. Two types of H$^+$-ATPase are involved in the acidification of internal compartments in *Trypanosoma cruzi*. *Biochem J 331*: 583-589.

Scott DA, Docampo R 2000. Characterization of isolated acidocalcisomes of *Trypanosoma cruzi*. *J Biol Chem 275*: 24215-24221.

Scott DA, De Souza W, Benchimol M, Zhong L, Lu H-G, Moreno SNJ, Docampo R 1998. Presence of a plant-like proton pumping pyrophosphatase in acidocalcisomes of *Trypanosoma cruzi*. *J Biol Chem 273*: 22151-22158.

Scott DA, Docampo R, Dvorak JA, Shi S, Leapman RD 1997. In situ compositional analysis of acidocalcisomes in *Trypanosoma cruzi*. *J Biol Chem 272*: 28020-28029.

Segura EL, Cura EN, Paulone I, Vasquez C, Cerisola JA 1974. Antigenic makeup of subcellular fractions of *Trypanosoma cruzi*. *J Protozool 21*: 571-574.

Segura EL, Vazquez C, Bronzina A, Campos JM, Cerisola JA, Gonzalez Cappa SM 1977. Antigens of the subcellular fractions of *Trypanosoma cruzi*. II. Flagellar and membrane fraction. *J Protozool 24*: 540-543.

Shapiro SZ, Webster P 1989. Coated vesicles from the protozoan parasite *Trypanosoma brucei*: Purification and characterization. *J Protozool 36*: 344-349.

Shih S, Hwang HY, Carter D, Stenberg P, Ullman B 1998. Localization and targeting of the *Leishmania donovani* hypoxanthine-guanine phosphoribosyltransferase to the glycosome. *J Biol Chem 273*: 1534-1541.

Sledge WE, Larson AD, Hart LT 1978. Costae of *Tritrichomonas foetus*: purification and chemical composition. *Science 199*: 186-188.

Soares MJ, De Souza W 1988. Cytoplasmic organelles of Trypanosomatids: a cytochemical and stereological study. *J Submicrosc Cytol Pathol 20*: 349-361.

Soares MJ, De Souza W 1991. Endocytosis of gold-labeled proteins and LDL by *Trypanosoma cruzi*. *Parasitol Res 77*: 461-468.

Souto-Padron, T, De Souza W 1982. Fine structure and cytochemistry of peroxisomes (microbodies) in *Leptomonas samueli*. *Cell Tiss Res 222*: 153-158.

Souto-Padrón T, De Souza W, Heuser J 1984. Quick-freeze, deep-etch rotary replication of *Trypanosoma cruzi* and *Herpetomonas megaseliae*. *J Cell Sci 69*: 167-178.

Souto-Padrón T, Gonçalves de Lima VMQ, Roitman I, De Souza W 1980. Fine structure study of *Leptomonas samueli* by the freeze-fracture technique. *Z Parasitenkd 62*: 145-157.

Steiger RF, Opperdoes FR, Bontemps J 1980. Subcellular fractionation of *Trypanosoma brucei* bloodstream forms with special reference to hydrolases. *Eur J Biochem 105*: 163-175.

Steiger RF 1973. On the ultrastructure of *Trypanosoma (Trypanozoon) brucei* in the course of its life cycle and some related aspects. *Acta Trop 30*: 64-168.

Sullivan MA, Olson CL, Winquist AG, Engman DM 1994. Expression and localization of *Trypanosoma cruzi* hsp60. *Mol Biochem Parasitol 68*: 197-208.

Svedberg T, Pedersen KO 1940. *The Ultracentrifuge,* Clarendon, Oxford.

Taylor MB, Berghausen H, Heyworth P, Messenger N, Rees LJ, Gutteridge, WE 1979. Subcellular localization of some glycolytic enzymes in parasitic flagellated protozoa. *Int J Biochem 11*: 117-120.

Timm SL, Leon LL, Pereira NM, de Souza W, Queiroz-Cruz M, Brascher HM, Lima AO 1980. Isolation of an enriched plasma membrane-subpellicular microtubule fraction of *Leishmania mexicana amazonensis. Mem Inst Oswaldo Cruz 75*: 145-155.

Urbina JA, Moreno B, Vieerkotter S, Oldfield E, Payares G, Sanoja C, Bailey BN, Yan W, Scott DA, Moreno SNJ, Docampo R 1999. *Trypanosoma cruzi* contains major pyrophosphate stores and its growth in vitro is blocked by pyrophosphate analogs. *J Biol Chem 274*: 33609-33615.

Vercesi A E, Moreno SN J, Docampo R 1994. Ca^{2+}/H^+ exchange in acidic vacuoles of *Trypanosoma brucei*. *Biochem J 304*: 227-233.

Vickerman K 1973. The mode of attachment of *Trypanosoma vivax* in the proboscis of the tsetse fly *Glossina fuscipes*: An ultrastructural study of the epimastigote stage of the trypanosome. *J Protozool 20*: 394-404.

Vickerman, K, Preston, TM 1976. Comparative cell biology of the kinetoplastid flagellates. In WHR Lumsden, DA Evans (eds), *Biology of the Kinetoplastida,* Academic Press, London.

Vickerman K, Tetley L 1990. Flagellar surfaces of parasitic protozoa and their role in attachment. In RA Bloodgood, *Ciliary and Flagellar Membranes*, Booknews, London.

Voorheis HP, Gale JS, Owen MJ, Edwards W 1979. The isolation and partial characterization of the plasma membrane from *Trypanosoma brucei*. *Biochem J 180*: 11-24.

Webster P 1989. Endocytosis in African trypanosomes. I. Three-dimensional structure of the endocytic organelles in *Trypanosoma brucei* and *T. congolense*. *Eur J Cell Biol 49*: 295-302.

Webster P, Fish WR 1989. Endocytosis by African trypanosomes. II. Occurrence in different life cycle stages and intracellular sorting. *Eur J Cell Biol 49*: 303-310.

Woods A, Sherwin T, Sasse R, Macrae TH, Maines AJ, Gull K 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J Cell Sci 93*: 491-500.

Woodward R, Carden J, Gull K 1994. Molecular characterization of a novel, repetitive protein of the paraflagellar rod in *Trypanosoma brucei*. *Mol Biochem Parasitol 67*: 31-39.

Wrightsman RA, Miller MJ, Saborio JL, Manning JE 1995. Pure paraflagellar rod protein protects mice against *Trypanosoma cruzi* infection. *Infect Immun 63*: 122-125.

Zingales B, Carniol C, Abrahamson PA, Colli W 1979. Purification of an adenyl cyclase-containing plasma membrane fraction from *Trypanosoma cruzi*. *Biochim Biophys Acta 550*: 233-244.