Trypanosomatids as Models for Basic Studies on the Structural Organization of Eukaryotic Cells [1]

Wanderley de Souza
Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, CCS-Bloco G, 21941-900, Rio de Janeiro, Brasil
[1] Based on the Opening Lecture. Third Brazilian Symposium on the Study of Monoxenic Trypanosomatids, Alfenas, Minas Gerais, Brazil, 20-22, August, 1999

Abstract

Trypanosomatids comprise a large number of species found in insects. They can be easily cultivated in axenic media, and even in chemically defined media. This short review points out the importance of trypanosomatids especially the monokenics, as biological models for the study of basic questions in cell biology. Emphasis is given (a) to the process of cell transformation, (b) to the association of surface macromolecules through a glycosylphosphatidylinnositol anchor, (c) to the compartmentalization of the glycolytic pathway in the glycosomes, (d) to the process of endosymbiosis, as a basic phenomenon in the biogenesis of cell organelles, (e) to the presence of an organelle rich in DNA, as is the case of the kinetoplast, (f) to the presence of a special organelle, the acidocalcisome, involved in the concentration of calcium and pyrophosphate, (g) to a special organization of cytoskeletal structures such as the sub-pellicular microtubules and the paraxial rod, and (h) to the presence of a highly polarized endocytic system.

KEY WORDS: Protozoa - Trypanosomatids - Cell transformation - Glycosome - Endosymbiont - Kinetoplast - Acidocalcisome - Sub-pellicular microtubules - Paraxial rod - Endocytosis

Introduction

The Trypanosomatidae family comprises a large number of species belonging to the following genera: *Trypanosoma*, *Leishmania*, *Herpetomonas*, *Phytomonas*, *Leptomonas*, *Crithidia* and *Blastocrithidia*. They are also part of a larger group of eukaryotic microorganisms belonging to the class **Kinetoplastidea**, that includes the orders **Bodonida** and **Trypanosomatida** (Cavalier-Smith, 1981). The order Bodonida comprises two genera: *Bodo*, which are free-living organisms, and *Cryptobia*, which includes pathogenic organisms of fishes. The order Trypanosomatida includes species that are highly infective for humans and animals, such as *Trypanosoma cruzi*, that causes Chagas disease, *Trypanosoma rhodesiense*, and *Trypanosoma gambiense* that cause the sleepness disease in Africa; *Trypanosoma brucei*, *Trypanosoma equiperdum* and *Trypanosoma equinum* that cause diseases in various animals, *Leishmania*, that causes several types of leishmaniasis in humans and animals, etc. However, there is a large number of species belonging to the order Trypanosomatida that are found only in insects and have been known as monoxenic or even as lower trypanosomatids. Several species of this later group of trypanosomatids have been the subject of intense investigation in the last twenty years due to the fact that they can be easily cultivated in chemically defined media. In addition, since they are not pathogenic for man, they constitute good models for the analysis of basic biological phenomena that occur with the pathogenic ones which usually are more difficult to obtain and to study. The main objective of the present short review is to emphasize the value of these trypanosomatids as models to study, from the cell biology point of view, some basic biological processes. It was not my intention to carry out a deep review of the available literature and only a few papers will be cited.

A System for the Study of the Process of Reversible Cell Transformation

One characteristic feature of trypanosomatids is to present a life cycle involving different forms, which have been generally considered as different developmental stages. Figure 1 shows light micrographs of Giemsa-stained smears where the forms usually found in monoxenic trypanosomatids are shown. In the case of species which present more complex life cycles, involving vertebrate and invertebrate hosts, the concept of developmental stage is more clearly defined. In these cases it is possible to distinguish forms that are able to

infect mammalian cells, as is the case of the trypomastigote form of *T. cruzi*, or forms that are able to divide. In the case of the monoxenic species we still not have clear parameters to define developmental stages. However, it is possible to identify different forms during the development of the trypanosomatid in the insect or in vitro, in axenic cultures.

The forms indicated in figure 1 are identified based on three basic criteria: (a) the general form of the cell; (b) the way the flagellum emerges from the flagellar pocket, and (c) the relative position of the nucleus and the kinetoplast.

The studies carried out with members of the genus *Herpetomonas* showed that one long form predominates in culture, with the kinetoplast localized in front of the nucleus and with the flagellum emerging in the central portion at the anterior region of the protozoan. These forms correspond to promatigotes (Fig. 2). It was also recognized that in some cultures, especially in old ones, other forms where the kinetoplast was located at the posterior region of the cell, and the flagellum also emerged from the central portion of the protozoan, were observed (Figs. 5,7,9,10). They correspond

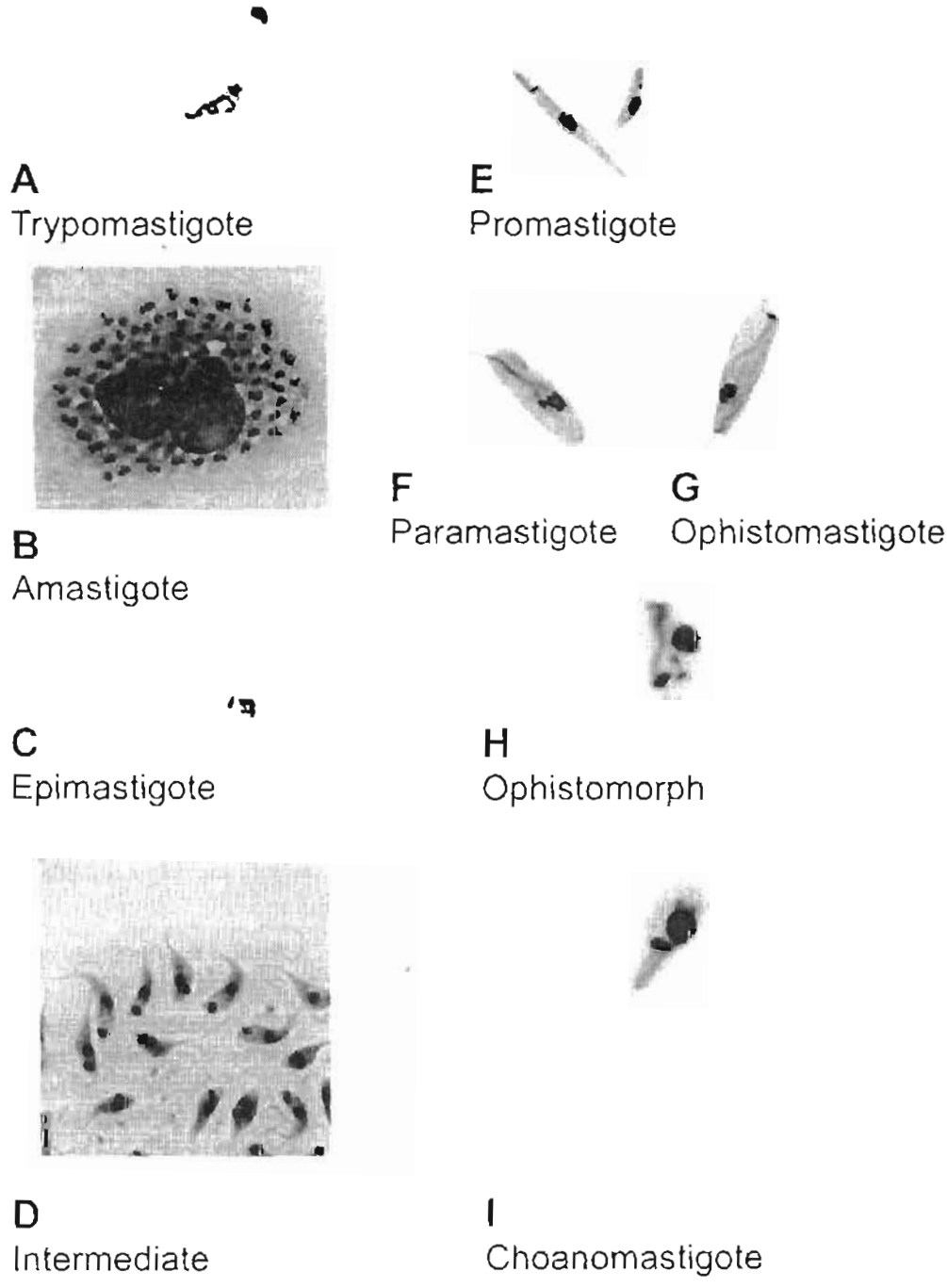
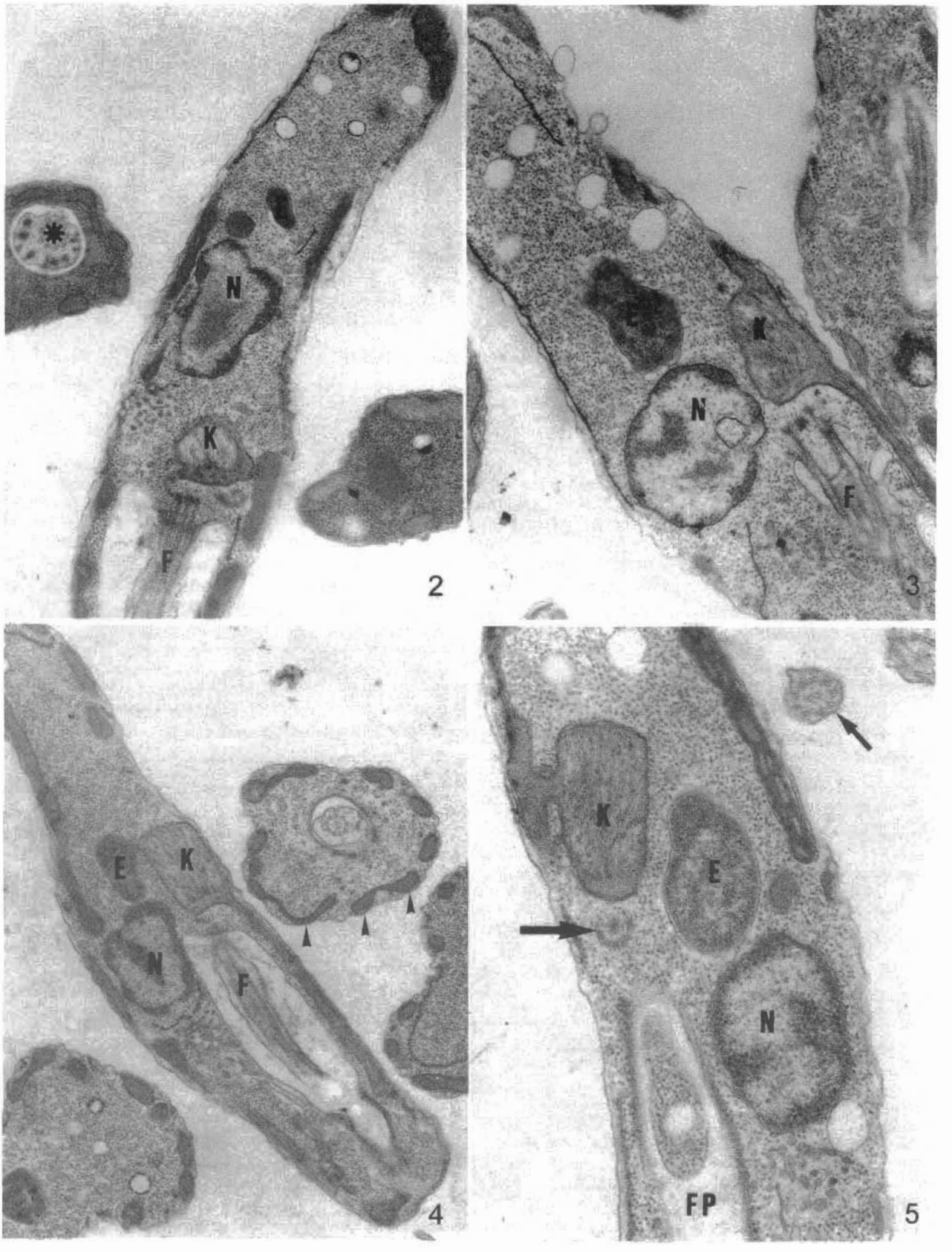
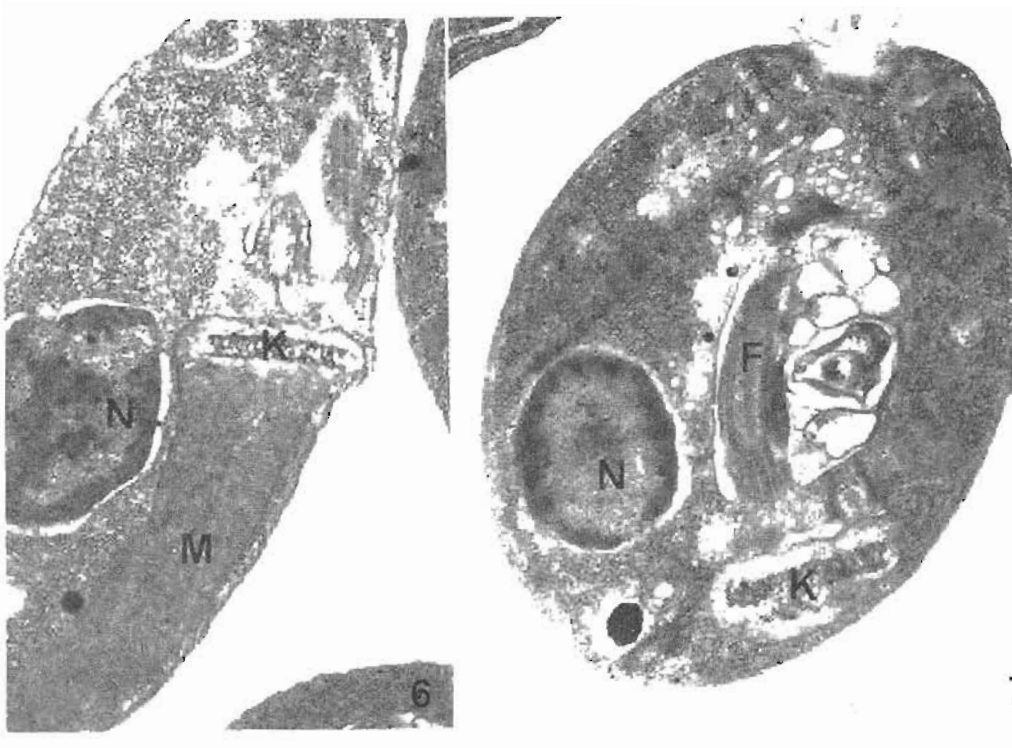


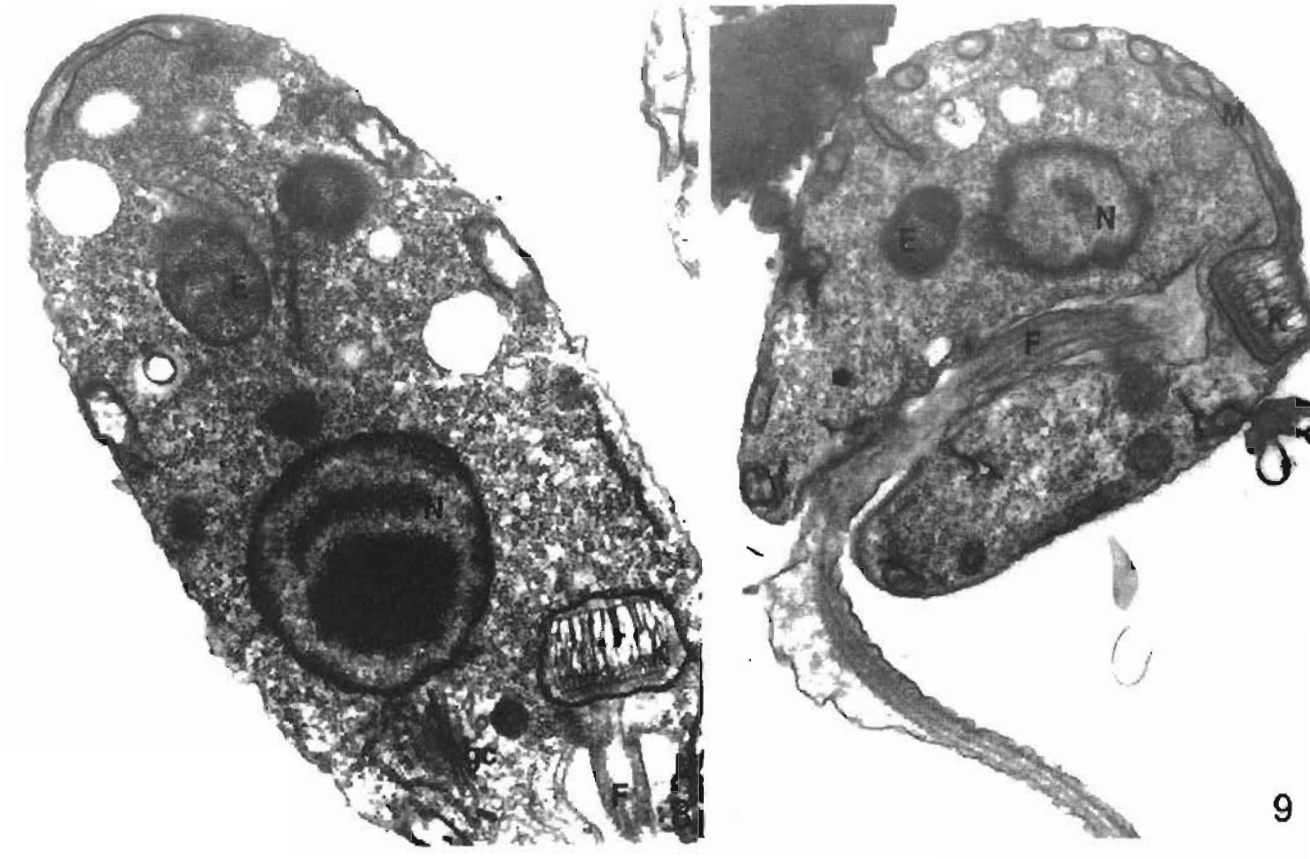
Figure 1. Micrographs of panotic-stained forms found in members of the *Trypanosomatidae family.*



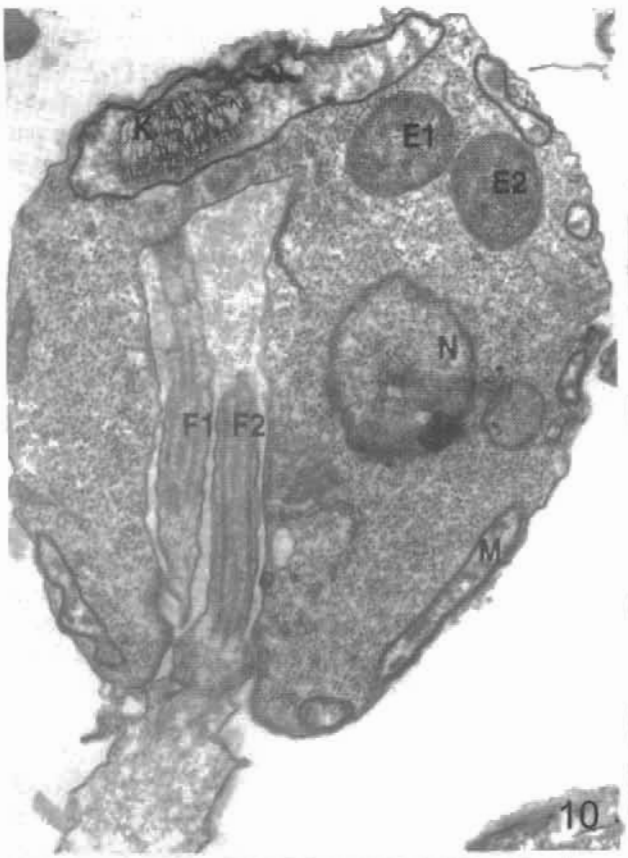
Figures 2-5. Transmission electron micrographs showing promastigote (Fig. 2), paramastigote (Figs. 3-4) and ophistomastigote (Fig. 5) forms of *Herpetomonas roitmani*. The typical kinetoplast (K) of endosymbiont containing species (E) is observed. The arrowheads in figure 4 point to peripheral branches of the mitochondrion. F, flagellum; N, nucleus. Figs. 2-3: X 21,000; Fig. 4: X 15,000; Fig. 5: X 30,000. (After Faria e Silva et al., 1991)

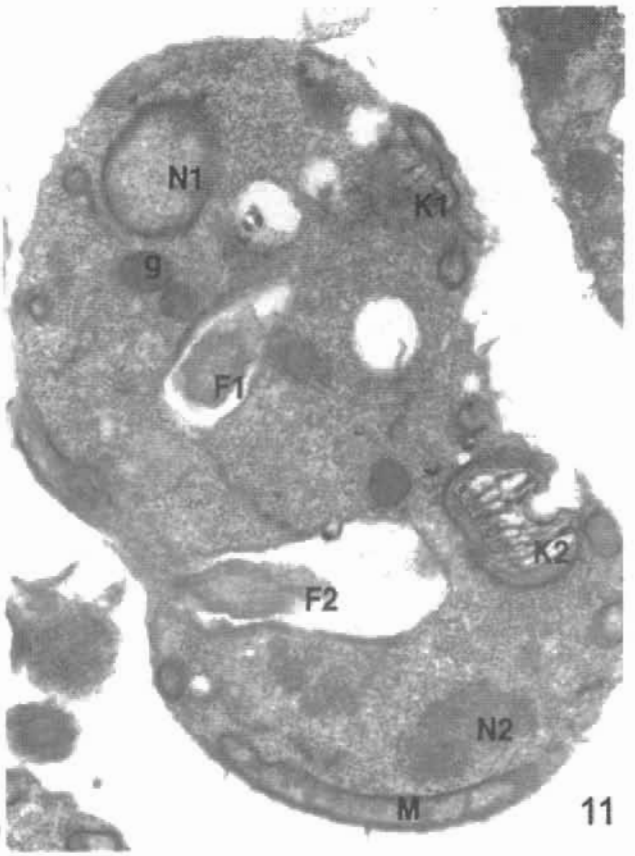


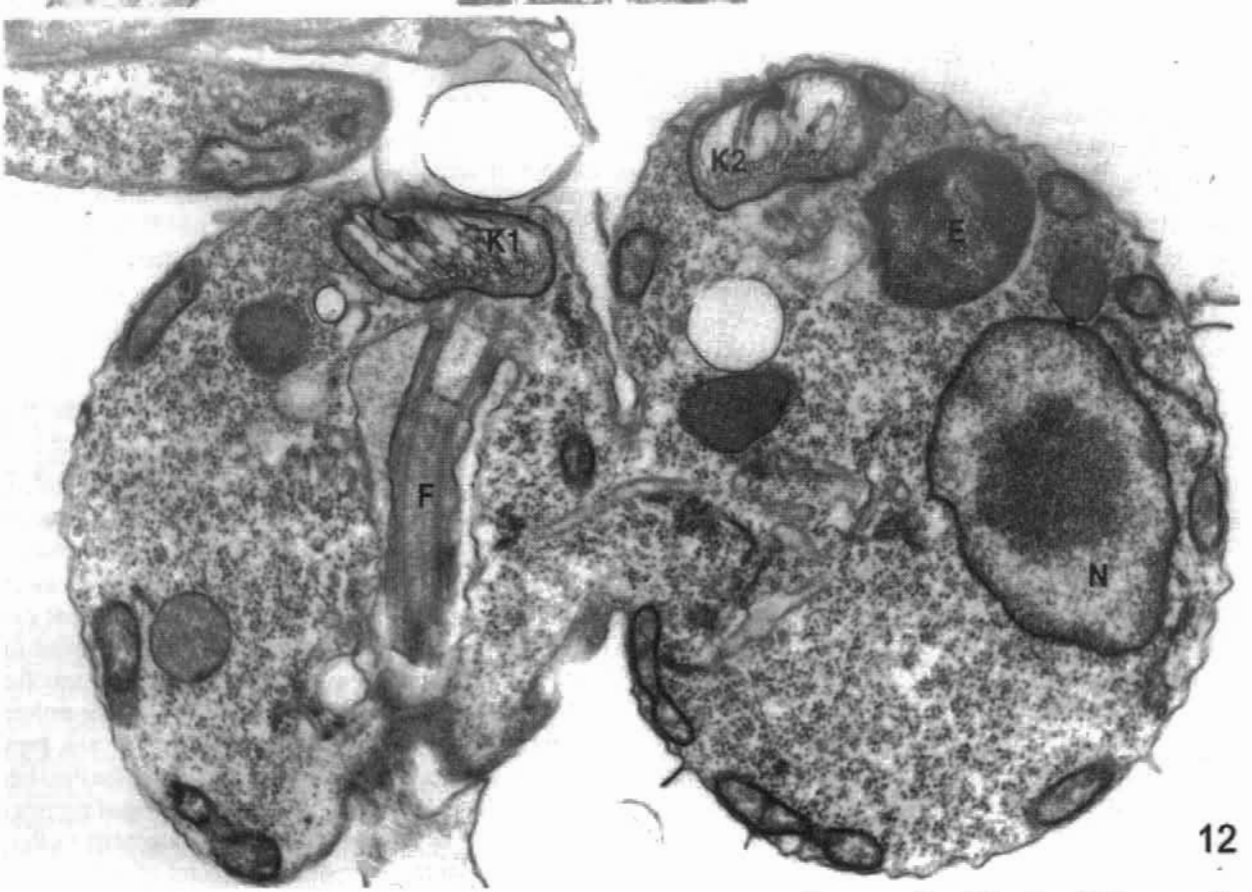
Figures 6-7. Thin sections showing the paramastigote (Fig. 6) and the rounded ophistomastigote (Fig. 7) form of *Herpetomonas samuelpessoai* grown in the presence of 2-deoxy-D-glucose. F, flagellum; K, kinetoplast; M, mitochondrion; N, nucleus. X 25,000. (After Angluster et al., 1977)



Figures 8-9. Thin sections showing the promastigote and the rounded ophistomastigote form of *Herpetomonas roitmani*, respectively. E, endosymbiont; F, flagellum; gc, Golgi complex; K, kinetoplast; M, mitochondrion; N, nucleus. X 36,000. (After Faria e Silva et al., 1996).







Figures 10-12. Ophistomastigote forms of *Herpetomonas roitmani* in process of division. E, endosymbiont; F, flagellum; K, kinetoplast; M, mitochondrion; N, nucleus. Figs. 10-11: X 36,000; Fig. 12: X 48,000. (After Faria e Silva et al., 1996).

to ophistomastigote forms and were characterized by transmission electron microscopy of *Herpetomonas megaseliae* in a seminal paper by Janovy and co-workers (Janovy et al., 1974). During many years this species was used as a model to study the process of **differentiation**, i.e., the transformation of promastigote into ophistomastigote. The possible similarity between this process and that observed in the genus *Trypanosoma* where epimastigotes transform into trypomastigotes was pointed out by several investigators. One characteristic common to both cases was that the proliferative forms found in cultures, promastigote for *Herpetomonas* and epimastigote for *Trypanosma*, had the kinetoplast located in front of the nucleus while the so called **differentiated** form had the kinetoplast localized behind the nucleus. This fact lead several groups to propose the system of transformation of promastigote into ophistomastigote forms in the genus *Herpetomonas* as a model to study the process of cell differentiation. This process has been widely studied in mammalian cells and the general concept which emerged considers differentiation when one cell passes through a series of well defined steps which lead to a final one in which it acquires specialized functions. Usually this process is irreversible and the differentiated cell is not able to divide anymore. As always, a few exceptions exist and a process of dedifferentiation has been described. Based on a few criteria, I prefer to consider the phenomenon observed in trypanosomatids a process of reversible transformation where, in some cases, the protozoan acquires special properties, including the ability to infect cells, of resisting to complement-mediated lysis, and even stoping dividing. However, a simple change in the environmental conditions may reverse the process.

The study of the process of transformation of promastigote into ophistomastigote forms in *Herpetomonas* has given important contributions to the better understanding of basic biological properties of this group of eukaryotic microorganisms. As pointed out before, the initial studies were carried out with *H. megaseliae* where it was shown that incubation of the protozoa in the presence of hydroxyurea induced the process of promastigote-ophistomastigote transformation, with the appearance of multiflagellated forms. One important observation in this area was reported by Roitman et al (1976) showing that the species *Herpetomonas samuelpessoai* was able to grow either at 28° or 37° C and that, at the higher temperature, the process of promastigote-ophistomastigote transformation was triggered. After the initial studies, *H. samuelpessoai* became the most used experimental model to study the process of reversible transformation in trypanosomatids. Indeed, several experimental systems were established in which the process of transformation was triggered: (a) it was shown that incubation of *H. samuelpessoai* in the presence of 2-deoxy-D-glucose, a carbohydrate analogue that interferes with the activity of several enzymes of the glycolytic pathway (Bunn et al., 1977), lead to a significant increase in the percentage of ophistomastigotes in the cultures maintained at 28° or 37° C. The cells became rounded and a large number of forms where the kinetoplast was located at the side of the nucleus was observed. These forms were named paramastigotes (Figs. 3,4,6) (Angluster et al., 1977). When cells were grown at 28°C for 92 hs in the presence of 30.5 mM of 2-deoxy-D-glucose, about 90 % of the cells were paramastigotes or ophistomastigotes; (b) incubation of *H. samuelpessoai* in the presence of different substances such as dimethylsulphoxide, concanavalin A, and lidocaine also lead to the process of promastigote-ophistomastigote transformation. It was considered that all treatments which interfere with components of the plasma membrane may trigger the transformation process.

In 1989, Fiorini and co-workers described three new isolates of trypanosomatids from insects found in Alfenas, Minas Gerais State, Brazil. Among them, one species initially designated as *Crithidia roitmani*, is of particular interest (Fiorini et al., 1989). This species was later on shown to correspond to a *Herpetomonas* which has the peculiarity of presenting a bacterium as endosymbiont in its cytoplasm (Faria e Silva et al., 1991). In this trypanosomatid long promastigotes, paramastigotes and ophitomastigotes could be observed (Figs. 2-5). After prolonged cultivation of *H. roitmani* in a chemically defined medium, it was noticed that cultures maintained at 28°C for 12 hs presented a larger number of ophistomastigote forms (Faria e Silva et al., 1996). Indeed, about 98 % of the cells presented a rounded form with the kinetoplast localized at the posterior region of the cell (Fig. 9). More interestingly, the ophistomastigotes were able to divide (Figs. 10-12). This observation is not in accordance with foregoing studies of transformation in members of the genus *Herpetomonas*, in which ophistomastigote forms have been found to appear in culture only at the end of the exponential phase of growth (Janovy et al., 1974; Roitman et al. 1976).

The Presence of Molecules Associated to the Plasma Membrane Through the GPI Anchor

One characteristic feature of trypanosomatids is to present a large number of macromolecules associated to the plasma membrane through an anchor of glycosylphosphatidylinnositol. This special type of membrane anchorage was first described for the variant surface protein which makes the surface coat of bloodstream forms of *Trypanosoma brucei* (Cardoso de Almeida and Turner, 1983). Later on, it was shown that many surface molecules, especially glycoproteins and lipophosphoglycans, found in lower trypanosomatids are also anchored to the lipid bilayer via GPI. This observation makes this group of microorganisms excellent models to study the biogenesis of the GPI anchor. From the morphological point of view molecules anchored via GPI can be detected by immunolabeling of freeze-fracture replicas, as recently shown for the localiza-

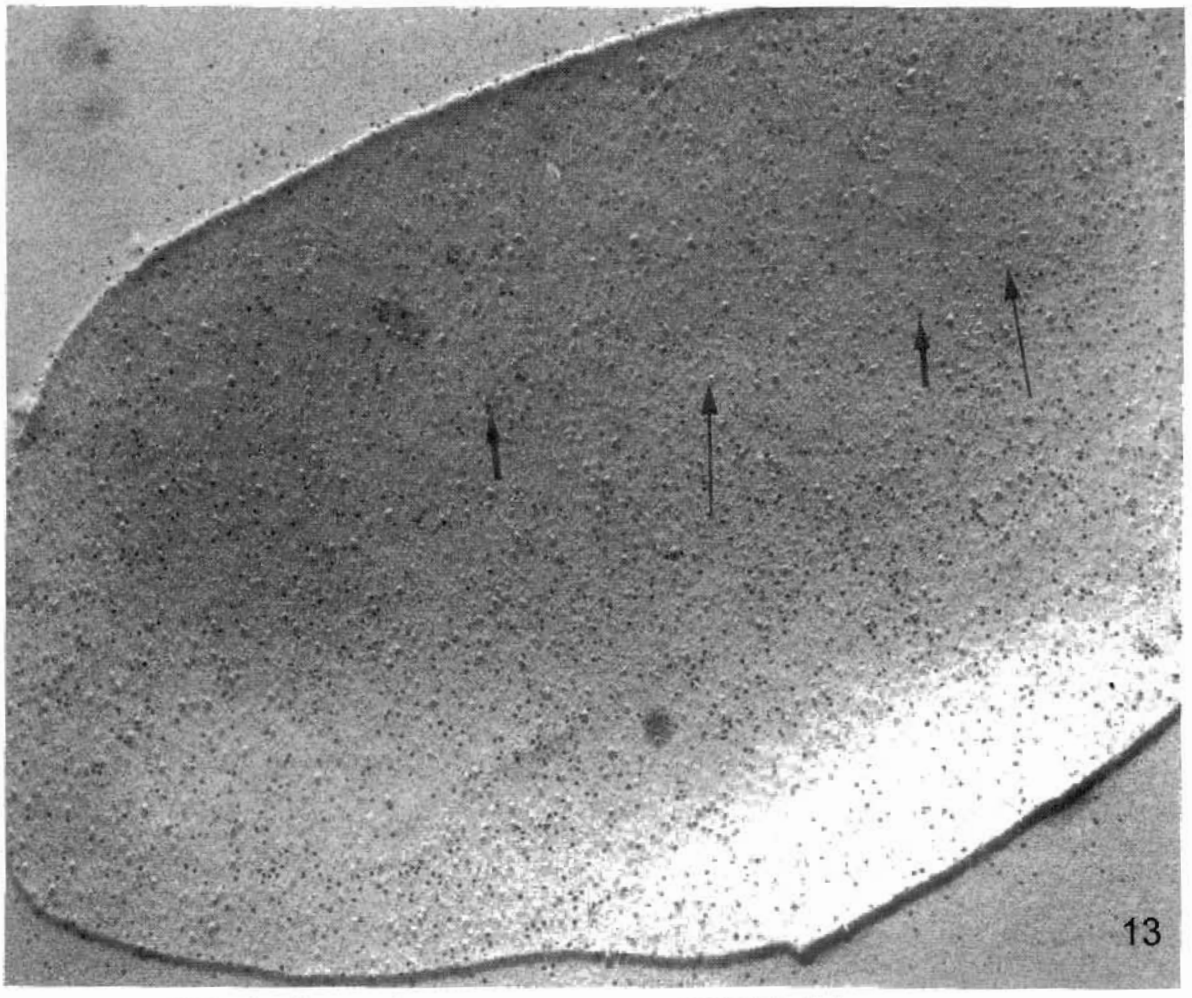


Figure 13. Label fracture replica of an amastigote form of *Trypanosoma cruzi* first incubated in the presence of a monoclonal antibody recognizing the Ssp4 and then in the presence of a secondary gold-labeled antibody. No relationship between the gold particles and the intramembranous particles was observed. X 63,000. (After Nascimento and De Souza, 1996)

tion of Ssp4 in amastigotes of *Trypanosoma cruzi* (Nascimento and De Souza, 1996). In this case no physical relationship between intramembranous particles, which correspond to integral membrane proteins, and collodial gold particles, used to reveal the surface macromolecule, exist (Fig. 13).

Advantage of the Compartmentalization of Metabolic Pathways: The Glycosome

The glycolytic pathway plays a fundamental role in the basic metabolism of cells. The various steps of this process, which involves the participation of several enzymes, takes place in the cytoplasm of the cells. In the case of protozoa of the Kinetoplastidea class, most of the glycolytic pathway takes place within a special organelle designated as the **glycosome**. This organelle was identified in the first studies analysing the fine structure of trypanosomatids by transmission electron microscopy. Usually it appears as a rounded structure enveloped by a typical unit membrane and presents a homogenous matrix (Figs. 14-16). At first it was designated as a microbody, a general term used to describe similar structures in other cells. In the case of mammalian cells some of the structures initially designated as microbodies were shown to contain catalase and other enzymes, and are now recognized as peroxisomes. Cytochemical studies carried out in some trypanosomatids also showed the presence of catalase activity in the matrix of the microbodies (Fig. 17), and therefore this organelle was considered as member of the peroxisome family (Review in Opperdoes and Michels, 1991). The application of the diaminobenzidine technique to detect catalase activity in trypanosomatids showed that the presence of this enzyme varies according to the species. In general no catalase activity has been cytochemically detected in similar structures found in trypanosomatids of the genus *Trypanosoma* and *Leishmania*. However, enzyme activity was observed in *Crithidia*, *Herpetomonas* and *Leptomonas*. Morphometric studies showed that the volume occupied by the glycosomes varies according to the developmental stages, especially in *T. brucei*, where such variation

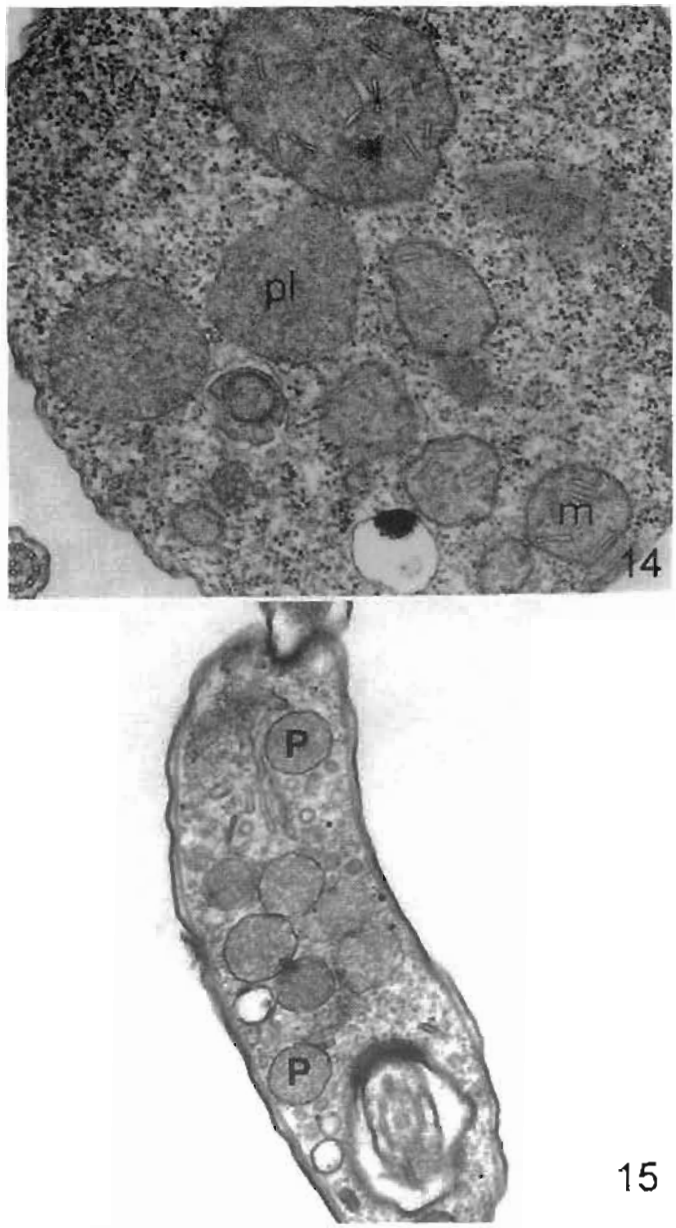
also correlates with the volume occupied by the mitochondrion. Bloodstream trypomastigotes do not present a clearly identified mitochondrion but present a large number of glycosomes. In contrast, during transformation of bloodstream trypomastigotes into epimastigotes in axenic cultures or in the insect, the mitochondrion outstands and the number of glycosomes decreases (Boehringer and Hecker, 1975). It was clearly shown that such changes are related with the general metabolism of the cell. In cells lacking the mitochondrion, an anaerobic metabolism predominates while in epimastigotes both aerobic and anaerobic metabolism take place.

Studies carried out with *Herpetomonas samuelpessoai* and *H. roitmani* showed that it is possible to interfere with the number of glycosomes varying the growth conditions. If the cells are grown in a chemically defined medium, containing sucrose as carbon source, there is a significant increase in the volume occupied by the glycosomes and reduction of the volume occupied by the mitochondrion. The reverse occurs if the cells are grown in a medium rich in proline (Faria e Silva et al., 1999). An interesting observation is that the total volume occupied by both organelles is always basically the same, around 10 %.

A comparative analysis of the glycosomes in several species show that some variation in their general shape occurs. In most of the species it appears as a spherical organelle distributed throughout the cell. In others, however, it may appear as a long organelle which can reach a length of 2.8 µm, as described in *Leptomonas samueli* (Souto-Padron et al., 1982). In some *Phytomonas* they are arranged in packs, while in others they are aligned (Attias et al., 1988).

Cytochemical studies aiming the localization of basic proteins using the ethanolic phosphotungstic acid technique showed an intense labeling of the glycosomes of all species analysed (Souto-Padron and De Souza, 1979) (Figs. 18-19). This staining pattern is basically due to the fact that the glycolytic enzymes found in the glycosomes of trypanosomatids present a high pHi.

The glycosome represents an interesting organelle from the evolutionary point of view. It is generally believed that cell compartmentalization represents an evolutive advantage, since a sequence of reactions takes place in a well defined compartment rather than dispersed in the whole cell. Analyzed from this perspective the glycosome is a remarkable model since the glycolytic pathway is one of the most important and well characterized metabolic routes found in eukaryotic cells.



Figures 14-15. Thin sections showing glycosomes (pl) in a promastigote of *Herpetomonas samuelpessoai* and in a bloodstream form of Trypanosoma brucei, respectively. M, mitochondrion. X 30,000. Fig. 14, after Souto-Padron and De Souza, 1979.

Endosymbiosis

Some members of the Trypanosomatidae family present a bacterium-like endosymbiont in the cytoplasm (Reviewed in De Souza and Motta, 1999). The endosymbiont can be seen by light microscopy of Giemsa-stained cells or of cells stained with any reagent which binds to DNA. The endosymbiont is localized close to the nucleus, presenting a length of 1.3 to 2.3 µm and a width of 0.3-1.0 µm (Figs. 2,5,8,9,20). Each cell harbors one or two endosymbionts, according to the phase of the cell cycle. The endosymbiont divides synchronously with the protozoan (Fig. 10) so that, at the end of the cycle, each daughter cell presents one endosymbiont.

The endosymbiont is surrounded by two unit membranes separated from each other by an electron lucent space of about 28 nm (Figs. 20-21). Freeze-fracture replicas have shown that there are differences in the density of intramembranous particles between the outer and the inner membranes

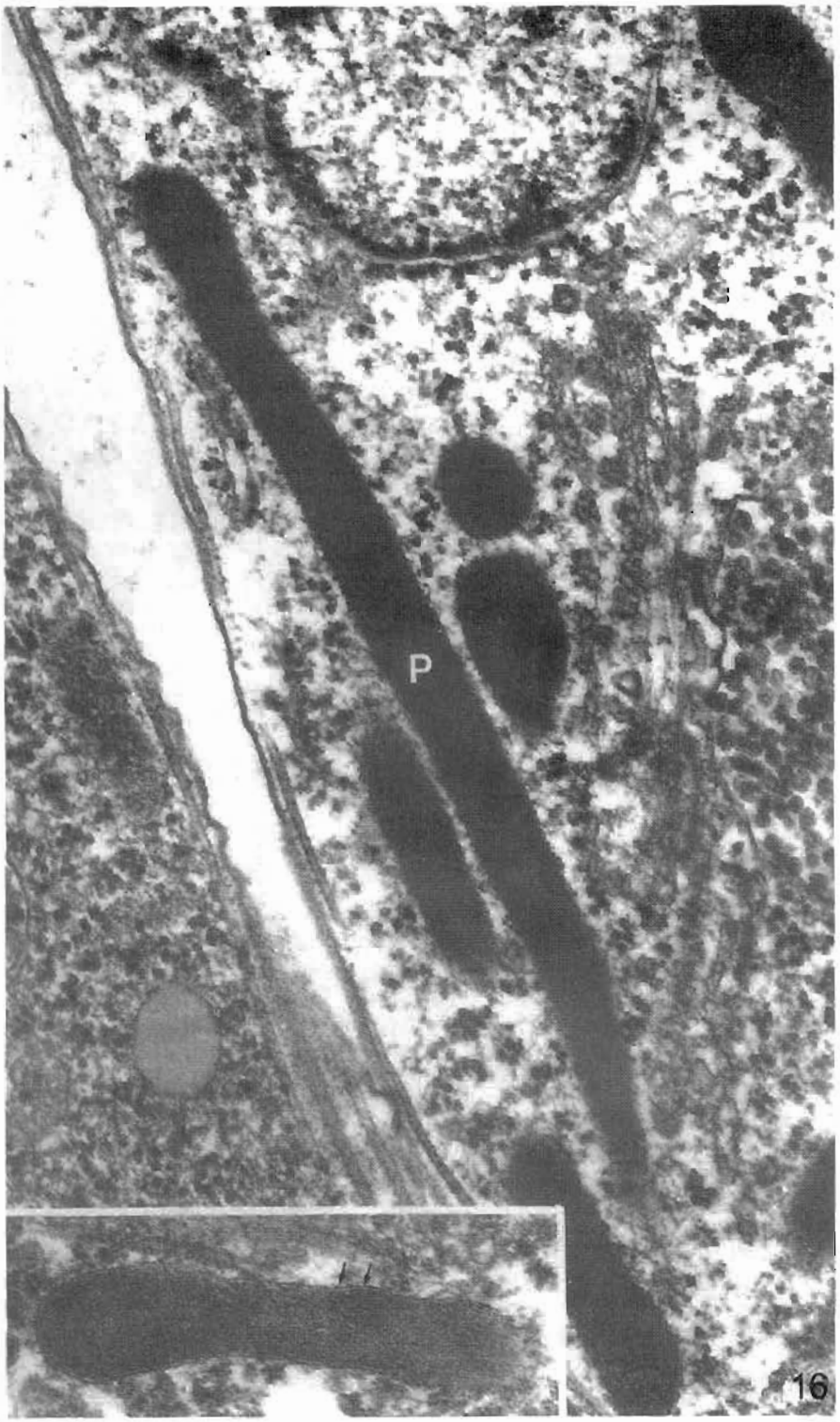


Figure 16. Thin section showing a long glycosome (P) in promastigote of *Leptomonas samueli*. X 60.000. Inset. X 99.000. (After Souto-Padron and De Souza, 1982)

(Fig. 22). The pattern of distribution of particles resembles that observed in Gram negative bacteria such as *Escherichia coli* (Soares and De Souza, 1988). No morphological evidence was obtained for the presence of a component equivalent to a cell wall or peptidoglycan layer in the endosymbiont. However, biochemical studies have shown that endosymbionts isolated from *Crithida deanei* by cell fractionation techniques showed the presence of two penicilin-binding proteins in the envelope of the endosymbiont (Motta et al., 1997). In addition, incubation of *C. deanei* in the presence of β-lactam antibiotics leads to dramatic changes in the morphology of the endosymbiont (Motta et al., 1997). The development of techniques to isolate the endosymbionts opens new possibilities for further studies.

The presence of the endosymbiont leads to dramatic alterations in the morphology of the trypanosomatids. These include: (a) the lack of the paraxial structure (also known as the paraflagellar rod), a complex array of filamentous structures which will be described below (Freymuller and Camargo, 1981); (b) a significant alteration in the morphology of the kinetoplast, as seen by transmission electron microscopy of thin sections. In all *Crithidia* the kinetoplast appears as a compact and electrondense structure while in endosymbiont-containing species two distinct regions of the kinetoplast can be identified. The anterior portion resembles that observed in other species while the most posterior region presents a more disperse arrangement of the DNA filaments (Figs. 3-5,8-12); (c) the presence of the endosymbiont also interferes with the spatial distribution of the microtubules and the mitochondrion. At some points of the cell surface, the sub-pellicular microtubules are displaced from their typical sub-pellicular localization and portions of the highly ramified mitochondrion touch the inner portion of the protozoan plasma membrane; (d) three-dimensional reconstruction showed that in endosymbiont-containing species the glycosomes concentrate around the endosymbiont thus suggesting some kind of metabolic integration between the two structures (Motta et al., 1997). Recent cytochemical studies showed that the type aa_3 cytochrome oxidase is inactive or absent in the endosymbiont respiratory chain. Similar results were obtained

for succinate dehydrogenase. These observations lead to the suggestion that part of the ATP produced in the mitochondrion and in the glycosomes of the protozoan is used by the endosymbiont, thus establishing a classic endosymbiotic process (Motta et al., 1997). Previous studies have shown that trypanosomatids harbouring an endosymbiont do not require a significant number of metabolites in the culture medium for their growth. These include hemin, purine, ornithine, arginine, citruline, leucine, isoleucine and valine, which are synthesized by the endosymbiont (Mundim and Roitman, 1977; Alfieri and Camargo, 1982; Newton, 1957; Mundim et al., 1974; Chang et al., 1975; Camargo and Freymuller, 1977).

Incubation of endosymbiont-containing trypanosomatids in the presence of chloramphenicol leads to the killing of the endosymbiont and the consequent obtaintion of strains which do not harbour the endosymbiont (Bruesk, 1967). Comparative analysis of strains of the same protozoa containing or not the endosymbiont have been carried and these constitute an excellent biological model to analyse the influence of the endosymbiont in the physiology of the cell.

One trypanosomatid species, designated as *Crithidia desouzai*, presents the interesting feature of harbouring simultaneously an endosymbiont and virus particles (Fiorini et al., 1989; Soares et al., 1989). Therefore, this single cell type presents four genomes: the nuclear DNA, the kinetoplast-mitochondrial DNA, the endosymbiont DNA, and the virus RNA (Fig. 23). The mechanisms which lead to the synchronization of the process of replication of these macromolecules is an interesting topic to be studied in the next years.

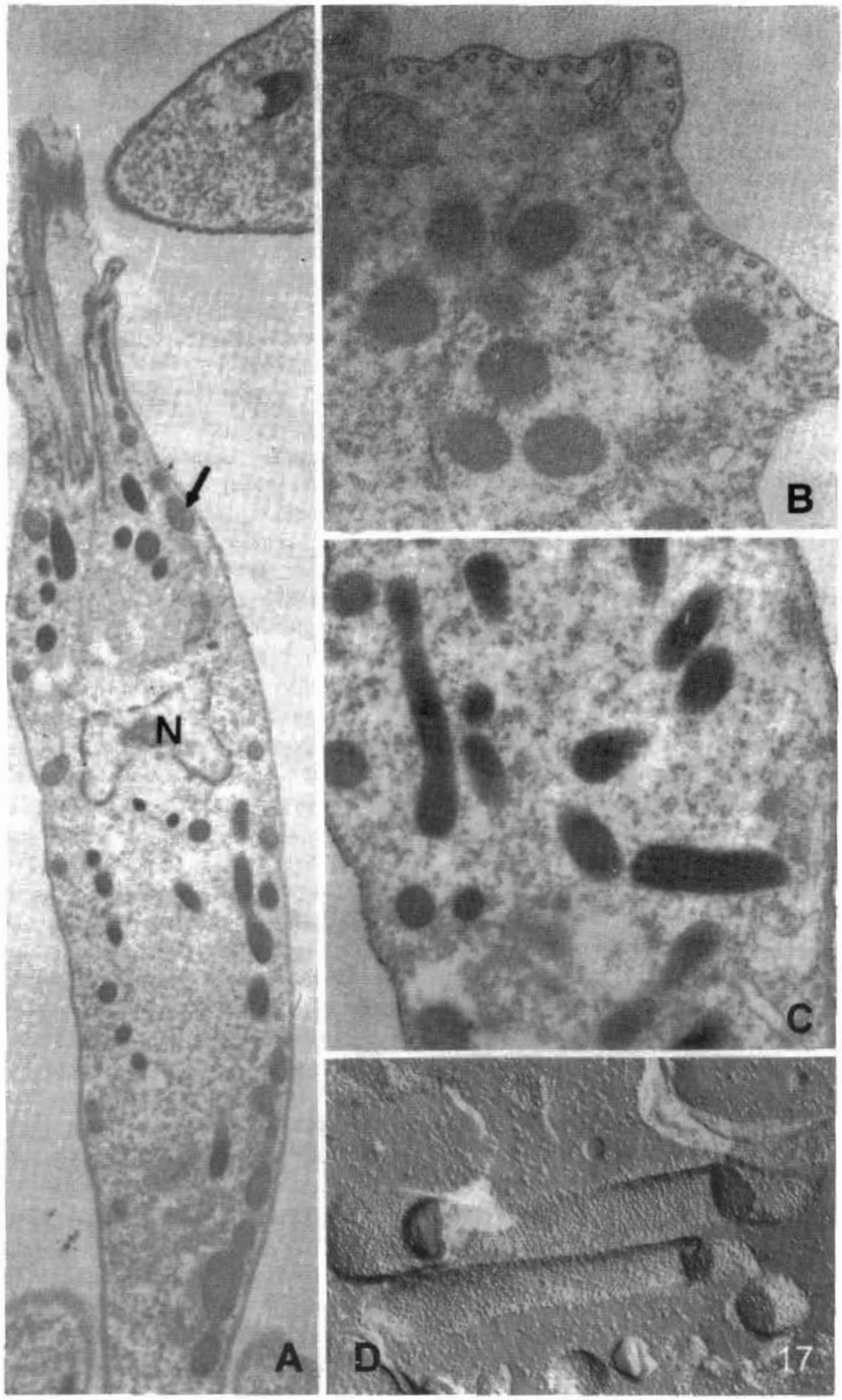
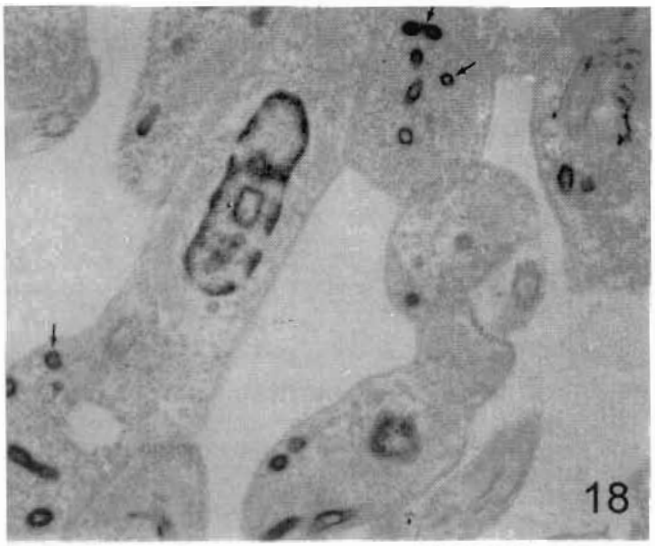
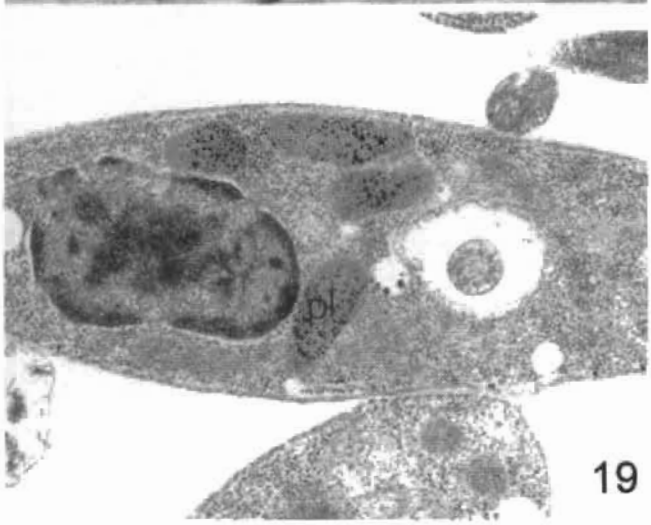


Figure 17. Different views of the glycosome of *Leptomonas samueli* incubated in the presence of a cytochemical medium designed for the localization of catalase, a marker of peroxisomes. Reaction product is seen in the organelle (A, C). No reaction is seen in a control preparation (B). D, snows a view of the organelle by freeze-fracture. A: X 13,700; B: X 48,000; C: X 30,000; D: X 37,500. (After Souto-Padron and De Souza, 1982).

It is well accepted that some organelles found in eukaryotic cells such as mitochondria and chloroplasts originated from microorganisms which established a symbiotic relationship with the host cell (Margulies, 1970; Cavalier-Smith, 1987). Some of the symbiont genes were possibly transferred to the host during the evolutionary process. Endosymbiont-containing trypanosomatids may constitute an excellent





Figures 18-19. Localization of basic proteins in the glycosomes of *Leptomonas samueli* and *Herpetomonas samuelpessoai*, respectively, as revealed using the ethanolic phosphotungstic acid technique. Fig. 18, unstained; Fig. 19, stained with lead citrate. Fig. 18: X 15,000; Fig. 19: X 30,000. (After Souto-Padron and De Souza, 1982).

model to study basic processes of the endosymbiont-host cell relationship and the origin of new organelles.

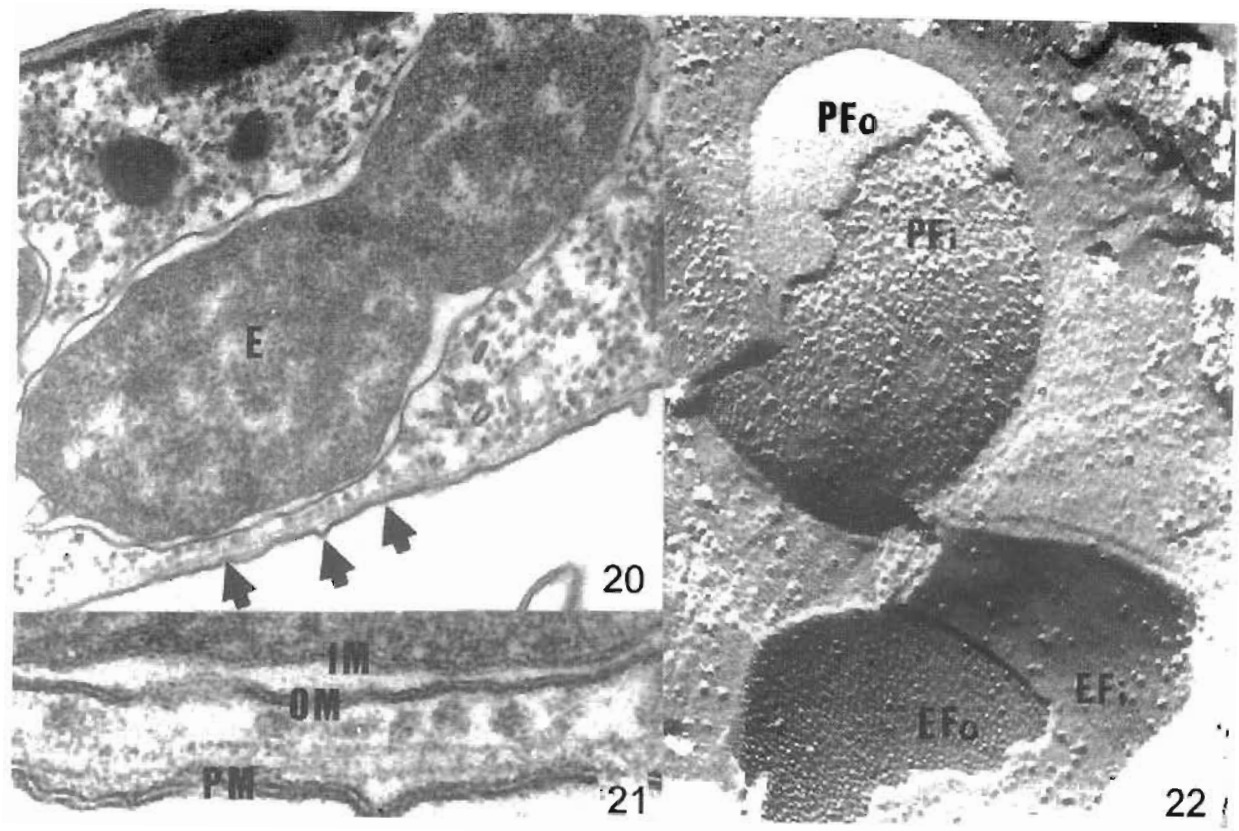
Synchronic Replication of DNA: The Kinetoplast

An intensely Giemsa-stained structure located close to the basal body from which the flagellum of trypanosomatids emerge was designated as the kinetoplast. The origin of the name is due to the assumption that the structure moved during the process of morphological transformation which takes place in the life cycle of these organisms. The analysis of the distribution of the sub-pellicular microtubules in amastigote, intermediate forms and trypomastigotes of *T. cruzi* lead to the suggestion that the kinetoplast does not move, but its relative position to the nucleus changes due to modifications in the arrangement of the sub-pellicular microtubules (Meyer and De Souza, 1974).

The interest in the kinetoplast starts when it was discovered that it is basically formed by DNA and localized in a well defined portion of the single and highly ramified mitochondrion found in trypanosomatids. This interest increased when it was shown that the DNA present in the kinetoplast may account for up to 30 % of the total DNA found in the cell. When good fixation of the cells was achieved, the fibrils which make the kinetoplast could be identified. Subsequently, with the isolation of the kinetoplast DNA, and its spreading on formvar- and carbon-coated grids, followed by rotatory shadowing at low angle, revealed that the kinetoplast DNA is composed of several thousand circular, covalently-closed, non-supercoiled DNA molecules catenated to form a huge planar network (Fig. 24). Two components were easily distinguished: (a) a huge number (about 20,000 to 30,000) of minicircles which have been shown to be heterogeneous in sequence but identical in size (length of 0.45 μm) within the network (Englund et al., 1996). It has been shown that they encode small guide RNAs which control the specificity of editing of maxicircle transcripts (Hadjuk and Sabatini, 1996). This process consists in the insertion or deletion of uridine, involving addition of few uridines as in the case of the mRNA coding for cytochrome oxidase II or addition of many uridines, and deletion of few uridines as in the case of the mRNA coding for cytochrome oxidase III in *T. brucei*; (b) maxicircles, present in several dozen, apparently identical, copies ranging from 20 to 38 kb and threaded through the catenated monolayer of minicircles. They have a length of 6 to 11 μm, which corresponds to the well known mitochondrial DNA found in all eukaryotic cells. The maxicircles encode rRNAs and mitochondrial proteins involved in functions such as electron transport and ATP synthesis. There is little information on the nature of the molecules involved in the stabilization of this complex structure. Cytochemical studies showed the presence of basic proteins associated to the kinetoplast. Indeed, a few kinetoplast proteins have been identified.

The replication of the complex array of catenated minicircles and maxicircles which make the kinetoplast has been intensely investigated in the last years. The results obtained have shown that minicircles are released from the network by the action of a topoisomerase II, and then, after replication, their progeny is reattached at antipodal positions on the network periphery. Two basic mechanisms of kinetoplast assembly have been reported. In one, the new replicated minicircles quickly become uniformly distributed at the network periphery, as reported for *C. fasciculata, Leishmania, T. cruzi* and *Crithidia luciliae*. In a second mechanism the minicircles accumulated near their two points of attachment, as occurs with *T. brucei* (Gullbride and Englund, 1998).

The studies described above have been carried out in trypanosomatids which present the so called rod-like kinetoplast. Transmission electron microscopy of thin sections have shown that there are other types of kinetoplasts, such



Figures 20-22. Different views of the membranes lining the endosymbiont of *Blastocrithidia culicis* as seen in thin sections (Figs. 20-21) and in freeze-fracture replicas (Fig. 22). IM, inner membrane of the endosymbiont; OM, outer membrane of the endosymbiont; PM, plasma membrane of the protozoan; Efo and Pfo are the extracellular and protoplasmic faces of the outer membrane of the endosymbiont; Efi and Pfi are the extracellular and protoplasmic faces of the inner membrane of the endosymbiont. Fig. 20. X 40,000; Fig 21. X 150,000, Fig. 22. X 64,000. (After Soares and De Souza, 1988).

as those found in trypomastigote forms of trypanosomatids of the *Trypanosoma* genus, *Schyzotrypanum* sub genus, as *T. cruzi, T. dionisii, T. vespertilionis*, etc. In this case, the DNA fibrils showed a more dispersed array or even a well organized basket-like form. A second type of kinetoplast is found in trypanosomatids which harbour an endosymbiont where a mixed type of kinetoplast is observed as described above. At present we still do not have information on the basic array of the minicircles and maxicircles in the last two types of kinetoplast.

New information on the organization of the kinetoplast DNA network was obtained in replicas of quick frozen, freeze-fractured, deep-etched and rotatory replicated cells. The kinetoplast appeared as formed by a complex array of polygonal, rather than circular, catenated structures. Fibrils connecting the network to the mitochondrial membrane and of the mitochondrion to the basal bodies could be visualized, thus explaining the proximity of the two structures (Fig. 24).

Regulation of Intracellular Calcium: The Acidocalcisome

It is now widely recognized that calcium plays a key role on the regulation of several cellular processes. Therefore, the eukaryotic cells developed a significant number of mechanisms and structures which are involved in the maintenance of a low concentration of calcium in the cytoplasm. Certainly, the endoplasmic reticulum and the mitochondria are the most important reservoirs of calcium within the cell. Recent biochemical and physiological studies carried out by Docampo and co-workers showed that the trypanosomatids, especially *T. brucei, T. cruzi* and *Leishmania*, present an additional reservoir with the additional feature that is an acidic compartment, which was then designated as the acidocalcisome (Docampo and Moreno, 1999; Docampo et al., 1995; Lu et al., 1998). Morphological studies, associating X-ray microanalysis and immunocytochemical localization of enzymes, showed that the acidocalcisome corresponds to a vacuole partially occupied by an electrondense material (Figs. 25-26). The preservation of the dense material, which contains calcium and phosphorus, varies according to the technique used to process the cells for electron microscopy. It is better preserved when cells are fixed using physical procedures such as high pressure freezing. The use of immunocytochemical techniques provided evidence for the presence of a H^+-ATPase in the membrane lining the acidocalcisome, thus explaining its acidic nature. A Ca^{2+}-ATPase and a Pyrophosphatase was

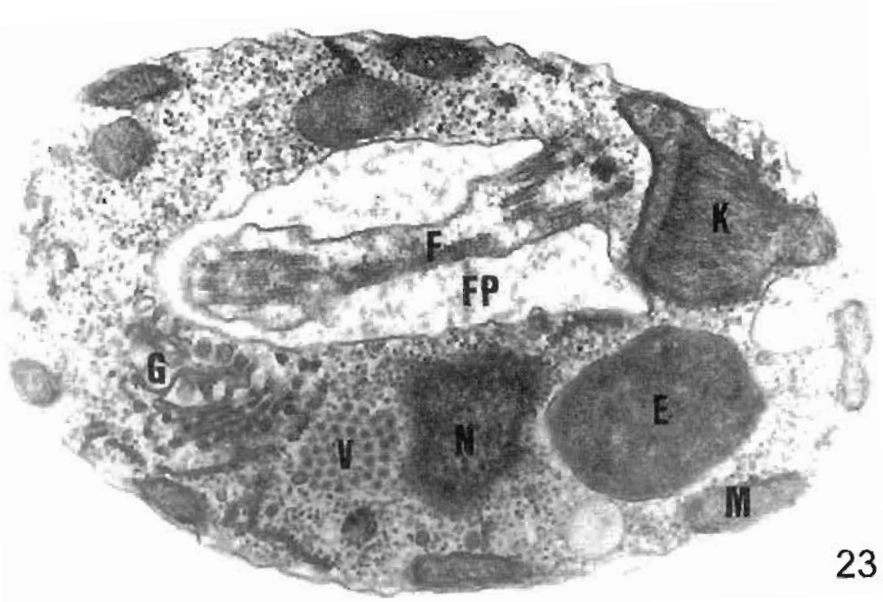


Figure 23. Thin section of *Crithidia desouzai* showing the kinetoplast (K), the endosymbiont (E), virus-like particles (V), Golgi complex (G), flagellum (F), mitochondrion (M), nucleus (N) and the flagellar pocket (FP). X 26.000. (After Soares et al., 1989).

also localized, making the acidocalcisome an interesting organelle from the cell biology point of view.

The number of acidocalcisomes in a species varies according to the developmental stage. In the case of *T. cruzi* there are much more acidocalcisomes in the amastigote form as compared with the epimastigote and trypomastigote forms. It is important to point out that the amastigote form lives in direct contact with the cytoplasm of the host cell which presents a very low concentration of calcium.

The available data on the acidocalcisomes are restricted to pathogenic trypanosomatids. However, morphological studies show that all trypanosomatids present a large number of organelles which resemble the acidocalcisome. Certainly this will be an area of further investigation in the next years.

A Special Cytoskeleton: The Sub-pellicular Microtubules and the Paraxial Structure

One characteristic feature of the trypanosomatids is the presence of an elaborated form of cytoskeleton which form two interesting systems: the sub-pellicular microtubules, and the paraxial structure (also known as paraflagellar rod).

Since the first observation of trypanosomes with the transmission electron microscope it was clear that these organisms presented a filamentous structure localized immediately below the plasma membrane (For a review see Gull, 1999), forming what was known as the periplast. The destruction of this structure by treatment of the cells with proteolytic enzymes revealed its proteic nature. The obtaintion of the first thin sections showed clearly that they corresponded to tubular structures, similar to those which make the flagellar axoneme. The microtubules are formed by 13 protofilaments, as revealed in cells fixed in tannic acid-containing glutaraldehyde solution, with a mean diameter of 24 nm. They form a helical pattern along the long axis of the cell, with a regular intermicrotubule spacing of about 44 nm. The microtubules are linked to each other and to the plasma membrane (Figs. 27-28) by filaments (Souto-Padron et al., 1984). The microtubules are of the stable type, containing acetylatedα-tubulin. They have an intrinsic polarity with the plus (+) ends pointing toward the posterior region of the cell. Although there are some information about microtubule-associated proteins we are far from a complete understanding of the biochemical composition and functional role played by proteins involved in the maintenance of the organization of the sub-pellicular microtubules in trypanosomatids. Since these microtubules are so abundant and stable, facilitating their study, it is expected that a larger number of investigators will work on this system in the next years. The sub-pellicular microtubules stabilize the protozoan and most probably drugs which disrupt it may constitute potential chemotherapeutic agents for treatment of diseases such as Chagas disease, African sleepness disease and leishmaniasis which have a high prevalence in several countries.

The second cytoskeletal structure that deserves a special comment is the paraxial structure (Bastin et al., 1996; Farina et al., 1986; Gull et al., 1999). In addition to the classical "9+2" microtubule axoneme, the flagellum of most trypanosomatids presents a lattice-like structure which runs alongside the axoneme as soon as the flagellum leaves the flagellar pocket (Figs. 29-34). The paraxial structure is formed by three distinct regions: proximal, intermediate, and distal. These regions can be easily recognized in cross sections of the axoneme, which also show that the paraxial structure is connected to the axoneme through axonemal doublets 4 through 7. The paraflagellar structure-axoneme connection is very stable, and is made of I or Y shaped filamentous structures. Mild trypsinization disrupts the connection, making possible the isolation of a highly purified fraction containing only the paraxial structure. Biochemical analysis has shown that the the paraxial structure is made of two major proteins, of 77 and 83 kDa, and several minor proteins. The functional role played by the paraflagellar structure has been studied in the last few years using a molecular genetics approach where it was possible to obtain null mutants of the major proteins. The data obtained show that the paraxial structure is involved in the normal flagellar motil-

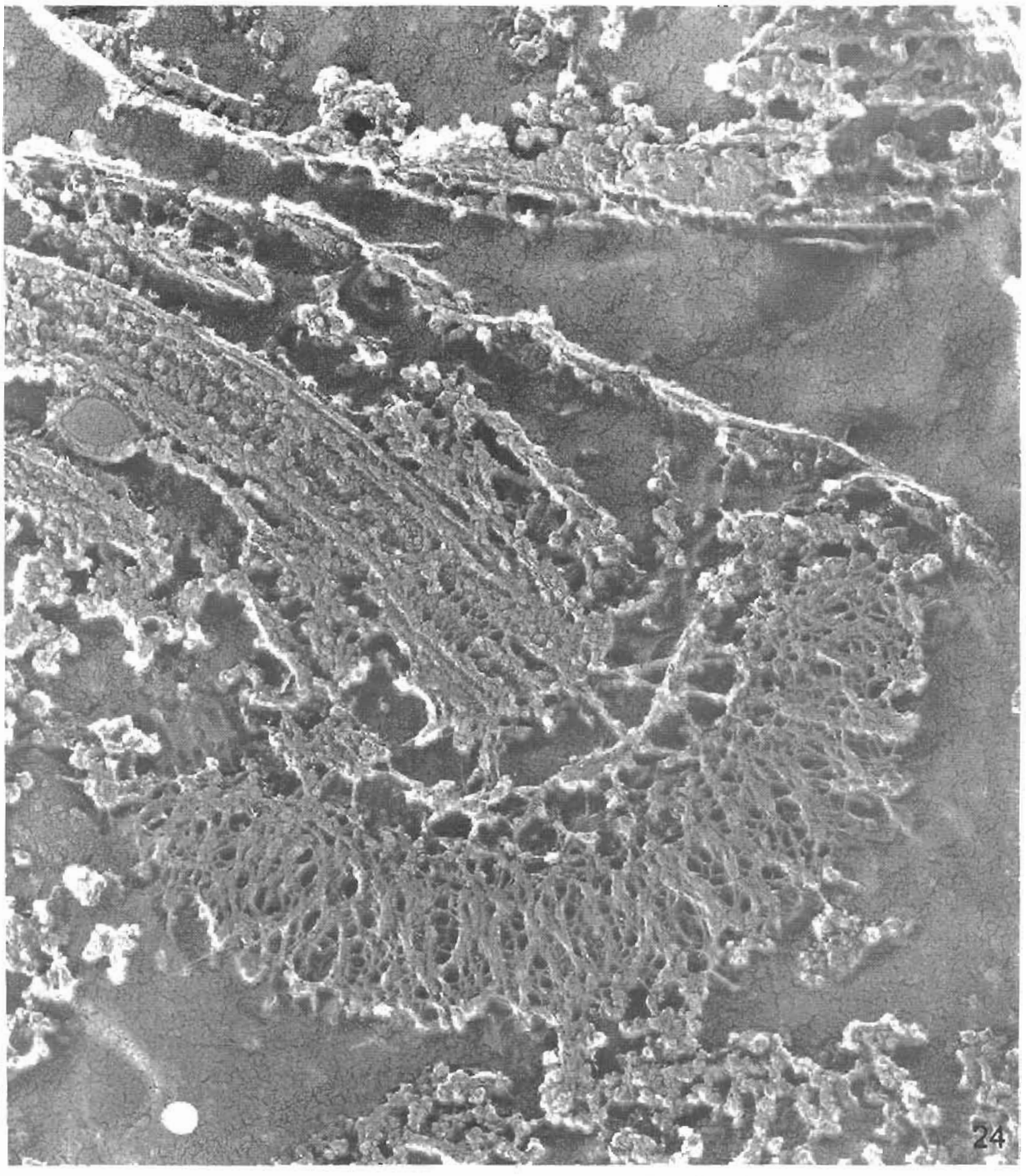
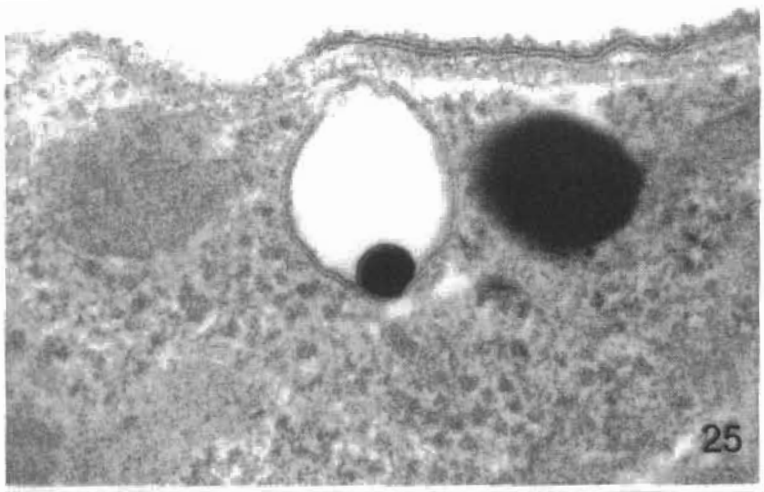
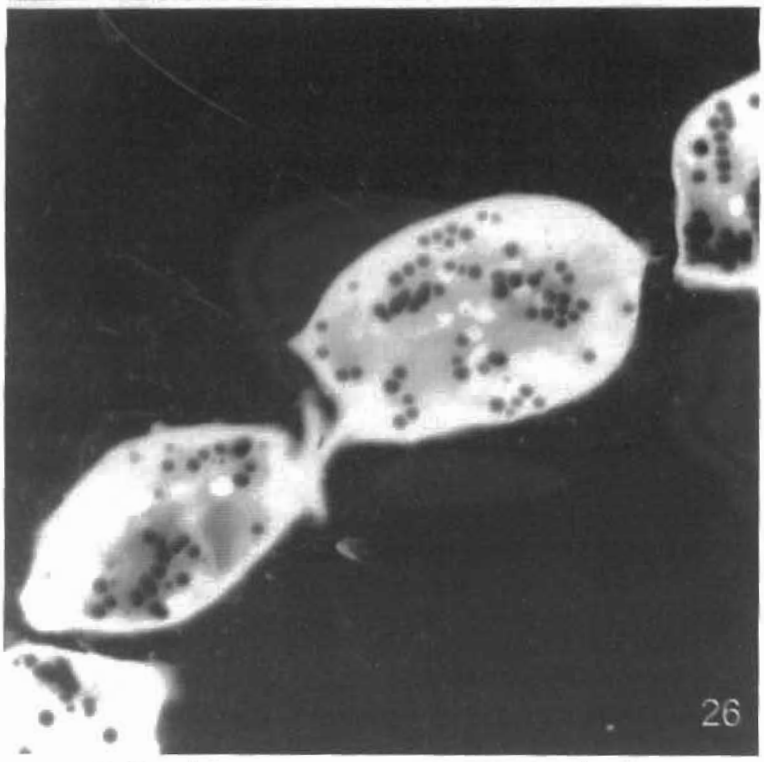


Figure 24. General view of the region of the kinetoplast and the emergence of the flagellum of an epimastigote form of *Trypanosoma cruzi* submitted to the quick freeze, freeze-fracture, deep-etching and rotary replication technique. X 102,000. (After Souto-Padron et al., 1984).





Figures 25-26. General view of the acidocalcisomes in a thin section (Fig. 25) and in the whole cell (Fig. 26) of *Trypanosoma cruzi*. In thin sections it appears as an empty organelle containing an electrondense material at the periphery. In the whole cell it appears as a homogenous electrondense organelle. Fig. 25. X 60,000 ; Fig. 26: X 15,000. (After Miranda et al., in press).

ity. However, further studies in this area are necessary since trypanosomatids which harbour a cytoplasmic endosymbiont do not have the paraflagellar structure, although they present a normal flagellar motility.

A Highly Polarized Endocytic Pathway

The trypanosomatids provide a system with special features for the study of the process of endocytosis, an activity extremely important to keep the cells alive and multiplying. In most of the isolated cells examined up to now endocytic activity, which can be morphologically characterized by the formation of small vesicles that pinch off from the plasma membrane, takes place in all regions of the cell. Only in organized tissues no formation of endocytic vesicles is observed in regions of cell-to-cell contact. In the case of confluent epithelial cells *in vitro* or true epithelium *in vivo*, endocytic activity takes place both in the apical and basal regions.

One characteristic feature of the process of endocytosis in trypanosomatids is that it only takes place in two well defined regions: the flagellar pocket and the cytostome.

The most important site of endocytic activity found in all species and in all developmental stages, is the flagellar pocket. It is formed by an invagination of the plasma membrane in the region of emergence of the flagellum, forming a true pocket (Webster and Russel, 1993). A close contact between the membrane lining the cell body and the flagellar membrane at the region where the flagellum leaves the pocket in some way controls the flux of material in and out the pocket. This contact region is characterized by the accumulation of a dense material below the membrane. This material is partially formed by basic proteins (Figs. 35-39), as revealed using the ethanolic phosphotungstic acid technique (Souto-Padron and De Souza, 1979), and by the presence of an aggregation of intramembranous particles (Fig. 40), as seen in freeze-fracture replicas. The region of the flagellar pocket does not present the layer of subpellicular microtubules and this explains the fact that endocytic vesicles can be formed only in the flagellar pocket. There are also evidence that vesicles formed in the Golgi complex moves in the direction of the flagellar pocket and fuse with its membrane. Therefore, the pocket is a site where endocytic and exocytic processes take place. Indeed, a large number of vesicles are always seen close to the pocket. Incubation of parasites in the presence of gold-labeled proteins reveal that the ligands are internalized through vesicles formed in the flagellar pocket. This has been well characterized in procyclic and bloodstream trypomastigote forms of *T. brucei*, and in *Leishmania*.

In the case of epimastigote forms of *T. cruzi* recent studies show that most of the endocytic activity takes place in the cytostome which is localized in the anterior portion of the cell, out of the flagellar pocket (Figs. 41-52). However, there is a specialized region of the protozoan surface which starts in the cytostome opening, and projects towards the flagellar pocket. The cytostome itself appears as a funnel-like invagination of the plasma membrane, lined by 5-10

microtubules. Endocytic vesicles bud off from the bottom of the structure which projects towards the nuclear region, reaching a length of up to 1.8 µm. In freeze-fracture replicas the opening of the cytostome appears as a specialized, smooth region of the plasma membrane, very poor in intramembranous particles, but delimited from the rest of the membrane by a pallisade-like array of particles. Surface glycoconjugates, which can be detected using gold-labeled lectins, concentrate at this region. A primitive cytostome, which appears as a bundle of a few microtubules running from the plasma membrane into the cytoplasm, has been observed in some lower trypanosomatids- choanomastigotes of the genus *Crithidia*, and promastigotes of *Leptomonas samueli*. However, there are no data showing ingestion of macromolecules through this cytostome.

The vesicles originated in the cytostome of epimastigotes of *T. cruzi* fuse to each other forming long tubular structures, which may reach a length of 2 µm, as revealed in three dimensional reconstruction of serial sections. These tubules may correspond to early endosomes (Fig. 47). Later on, there is the formation of circular structures, known as reservosomes, which are localized in the posterior region of the cell (Figs. 48-52). All macromolecules ingested through endocytic vesicles concentrate in the reservsome, which may account for up to 6 % of the total volume of the cell. The reservosome is an acidic compartment, with a pH about 6.0, and has been identified as a pre-lysosomal compartment. It disappears during the process of transformation of epimastigote into trypomastigote forms.

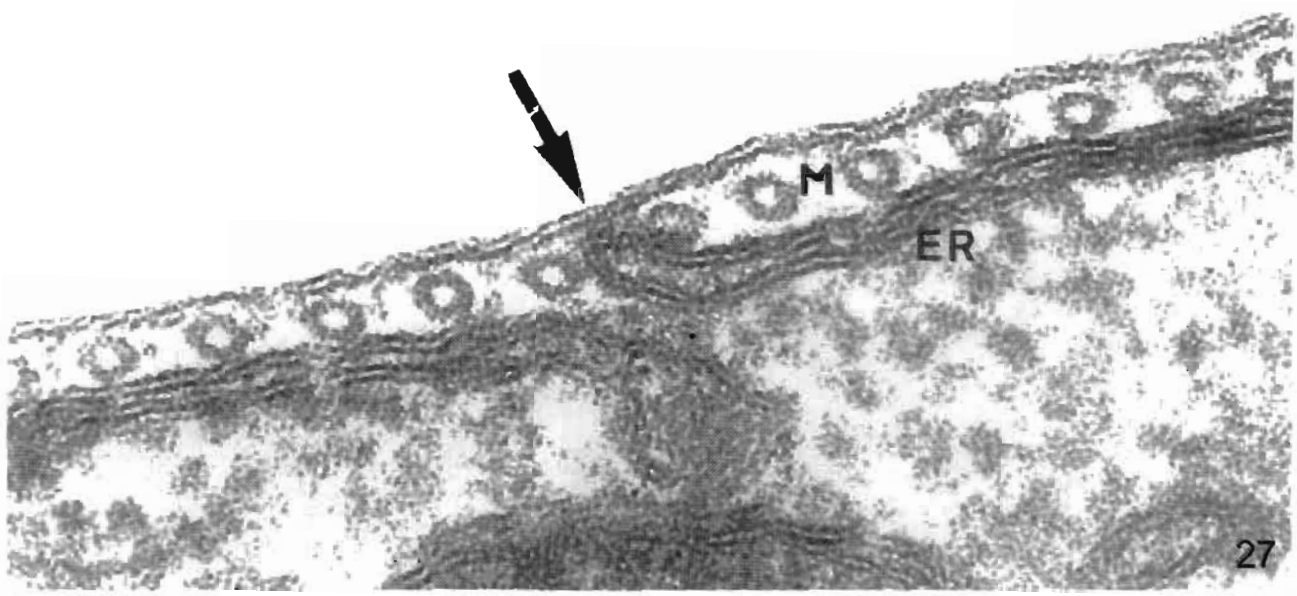


Figure 27. Thin section showing the close association of the sub-pellicular microtubules and profiles of the endoplasmic reticulum with the plasma membrane (arrow). X 170,000 (After Pimenta and De Souza, 1985).

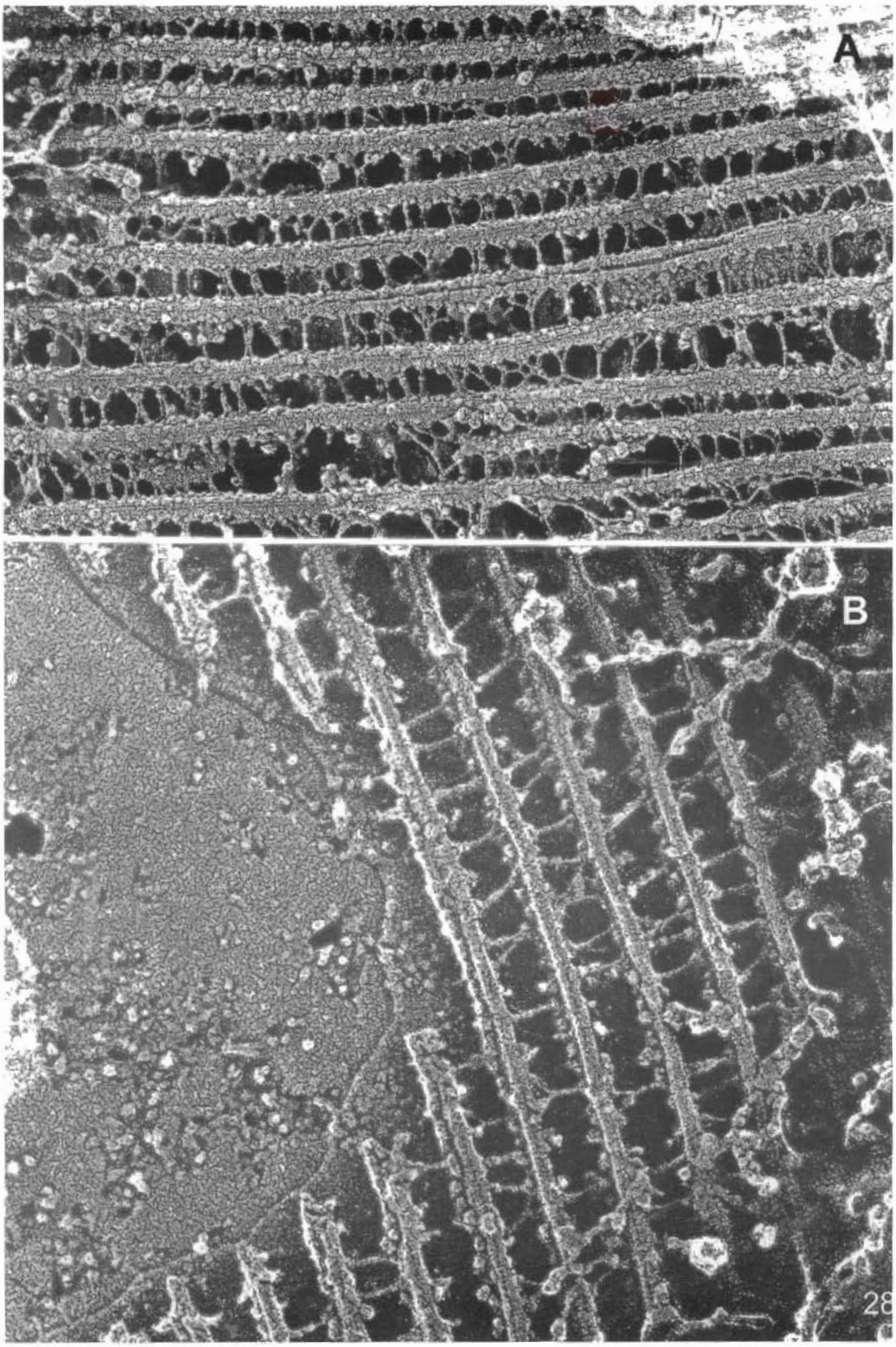
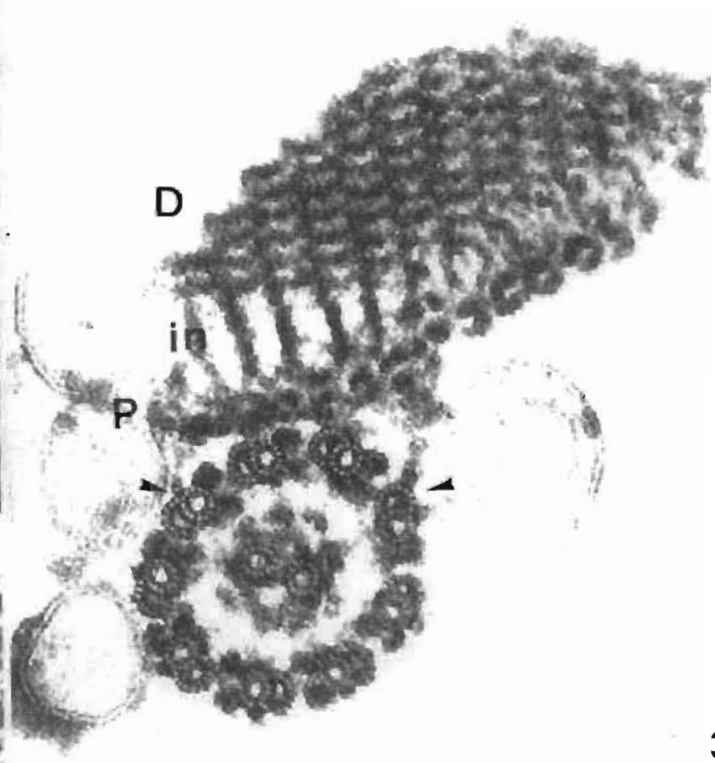
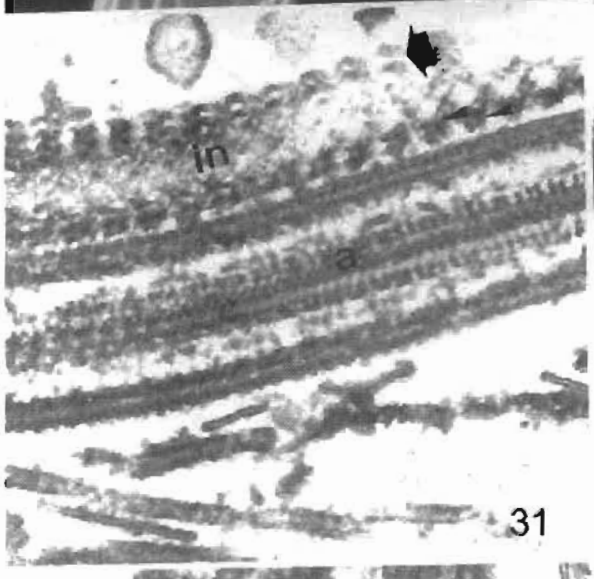
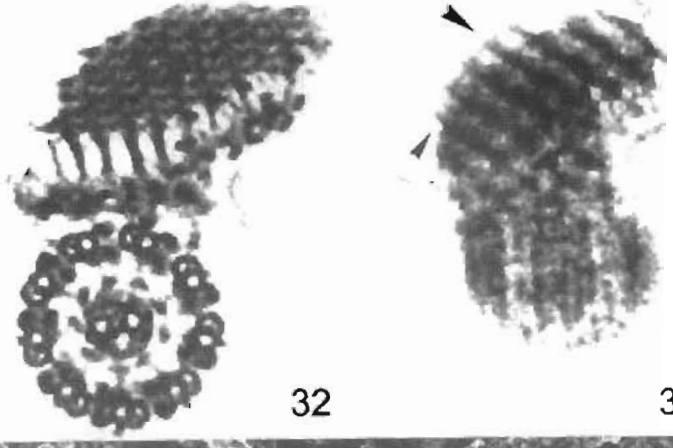
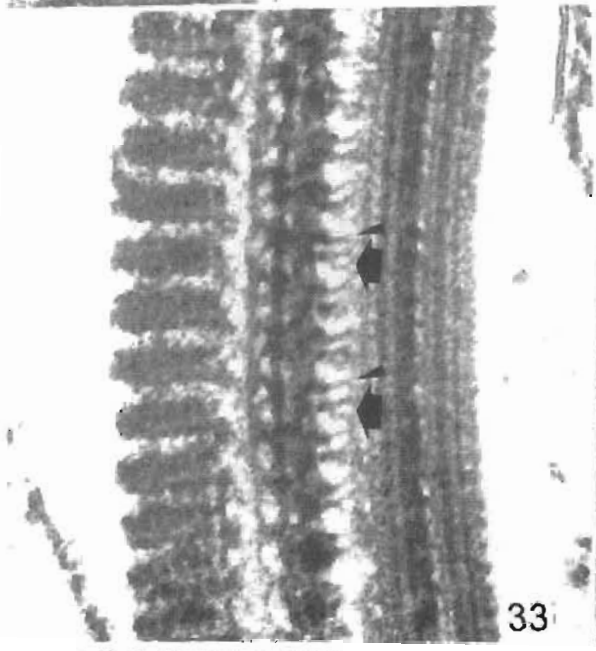


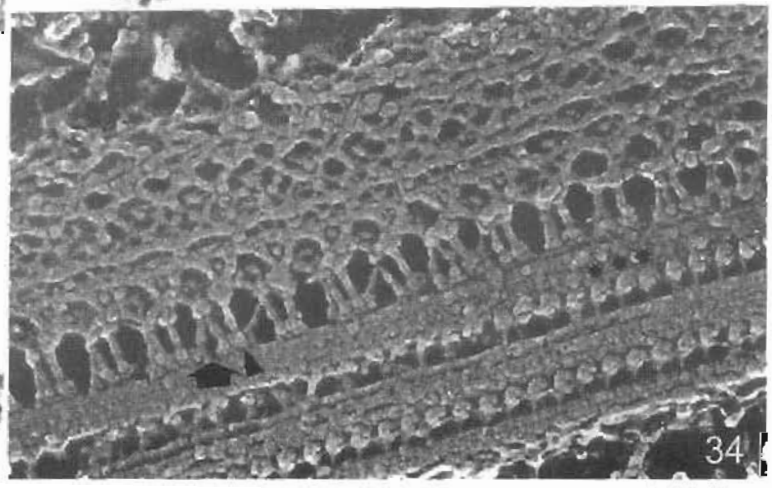
Figure 28. General views of the sub-pellicular microtubules of *Herpetomonas megaseliae* as visualized in replicas of quick frozen, freeze-fractured, deep etched and rotary replicated cells. Filamentous bridges which connect the microtubules can be seen. X 120,000



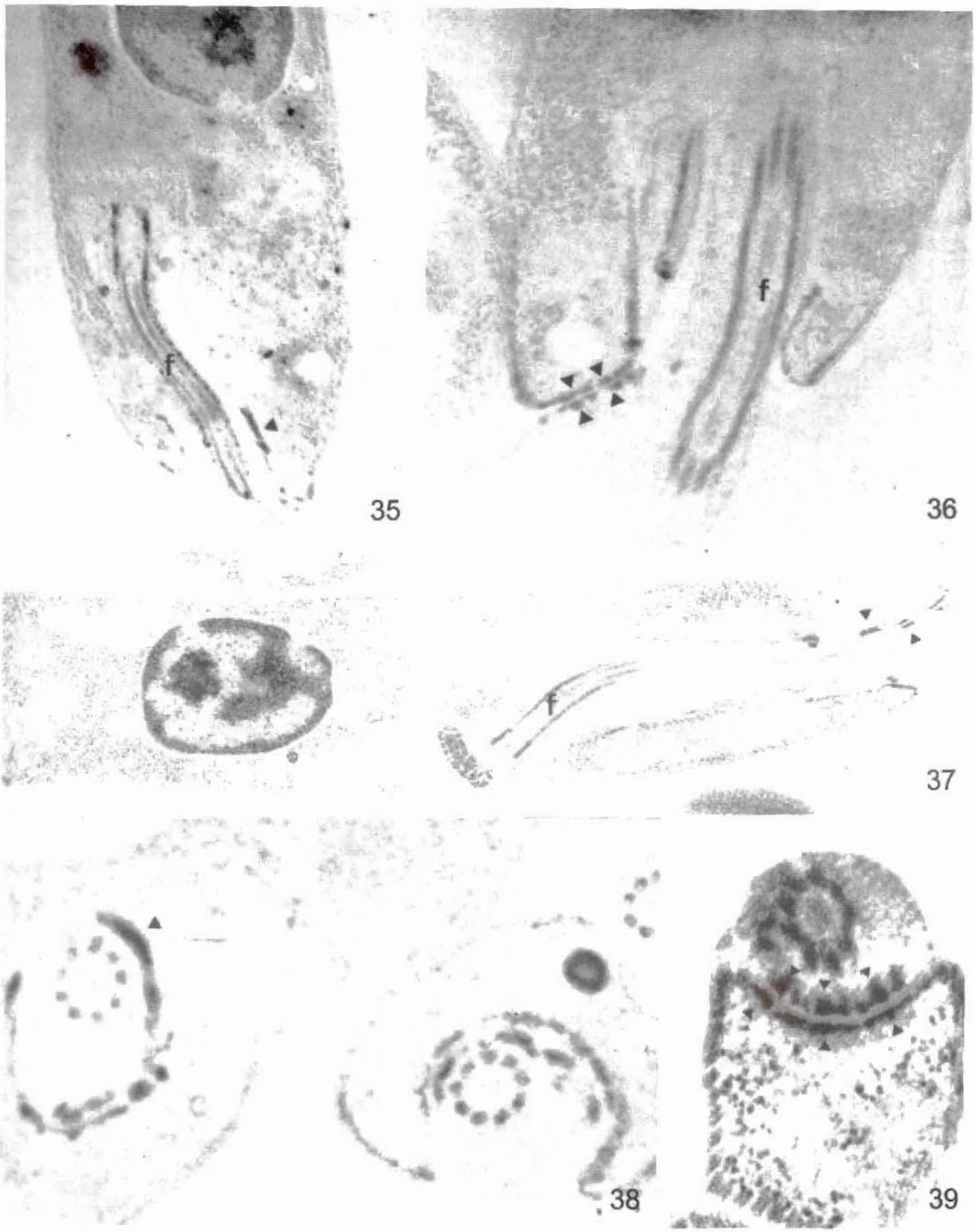








Figures 29-34. Electron microscopy of the flagellum and paraxial rod of *Herpetomonas megaseliae* (Figs. 29 and 34) and Phytomonas davidii (Figs 30-33) as seen by scanning electron microscopy (Fig. 29), thin sections (Figs. 31-33) and freeze-fracture (Fig. 34). The proximal (P), intermediate (in) and distal (D) regions of the paraxial rod are indicated. Thick arrows in figures 33 and 34 show bridges which connect the axoneme to the paraxial rod. Fig. 29: X 18,000; Fig. 30: X 160,000; Figs. 31, 33 and 34: X 100,000; Fig. 32: X 118,000. (After Farina et al., 1986).



Figures 35-39. Trypanosomatids submitted to the ethanolic phosphotungstic acid technique aiming the localization of basic proteins, which appear as electrondense regions areas. The arrowheads point to stained regions which are especially evident at the region of attachment of the flagellum to the cell body. F, flagellum. Fig. 35: X 30,000; Fig. 36: X 45,000; Fig. 37: X24,000; Figs. 38-39: X 60,000. (After Souto-Padron and De Souza, 1979).

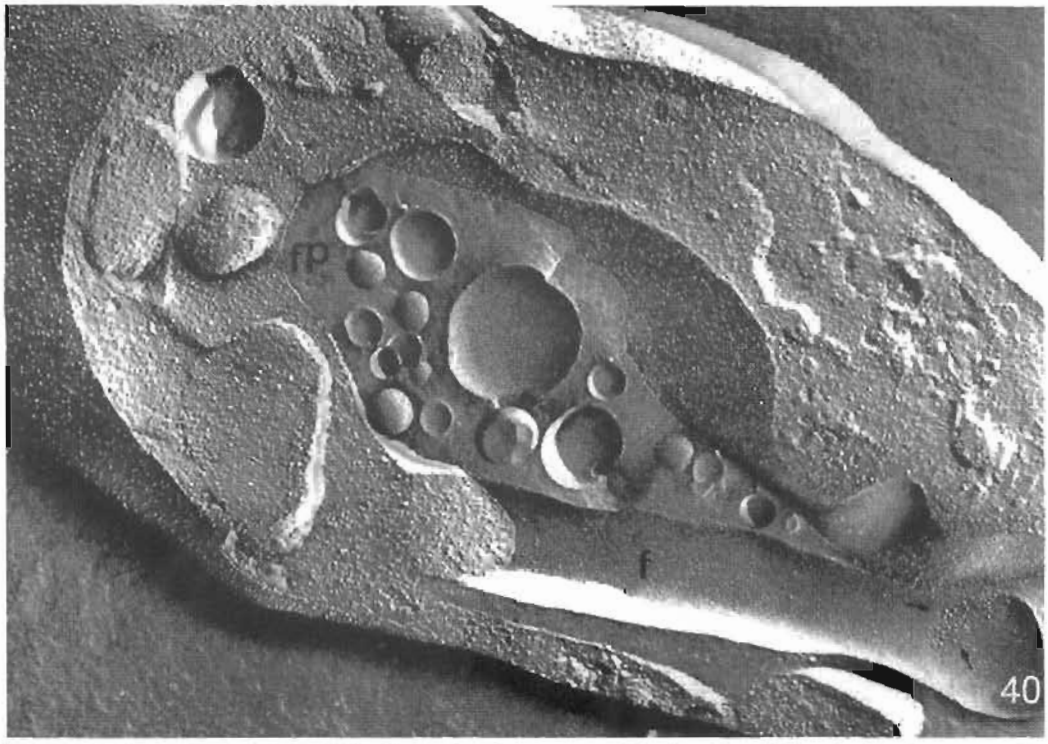
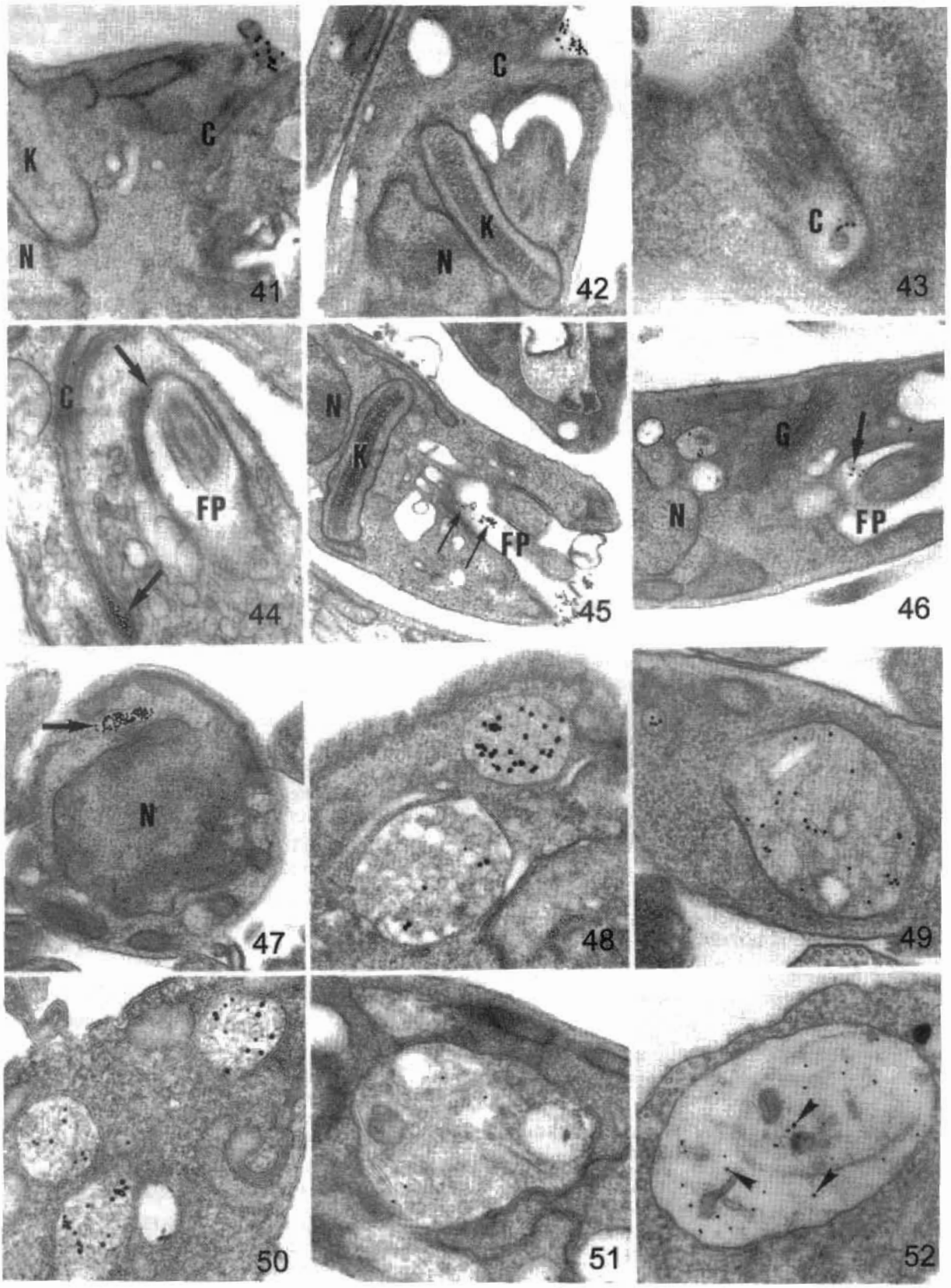


Figure 40. Freeze-fracture replica showing the region of emergence of the flagellum (F) from the flagellar pocket (FP) region of the promastigote form of *Herpetomonas samuelpessoai*. An aggregation of intramembranous particles (small arrows) is seen in the region of flagellum-cell body attachment. X 35,000. (After De Souza et al., 1979).



Figures 41-52. Different views of the endocytic pathway in epimastigote forms of *Trypanosoma cruzi* incubated in the presence of gold-labeled probes (proteins and lipoproteins). The probes are first observed in the cytostome and flagellar pocket region (Figs. 41-46). Subsequently are observed in tubules (Fig. 47) and then concentrate in the reservosomes (Figs. 48-52) which are localized in the posterior region of the protozoan. Figs. 41,42, 44, 46, 47: X 30,000; Fig. 43, 50: X 58,000; Fig. 45: X 23,000; Figs. 48,49, 51: X 40,000, Fig. 53: X 46,000. (After Soares and De Souza, 1991).

Acknowledgement

The work carried out in the author's laboratory has been supported by Programa de Núcleos de Excelência (PRONEX), Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), National Institutes of Health (NIH), and European Community.

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