INVITED REVIEW

Heart Muscle Cell and *Trypanosoma cruzi*: Mitochondrial Enzyme Cytochemistry and Immunolabeling

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Summary

This paper reviews our research on the localization of mitochondrial enzymes, using both cytochemistry and immunocytochemistry , in an experimental system of normal and **Trypanosoma cruzi** infected heart muscle cells (HMC). Using cytochemical methods, enzymes involved in the oxidation-reduction cycle - in particular those participating in the electron transport-such as cytochrome oxidase, succinate dehydrogenase (SDH), isocitrate dehydrogenase (ICDH), glycerophosphate dehydrogenase (GPDH), ß hydroxybutyrate dehydrogenase (ß HBDH) and NADPH diaphorase were localized on the inner mitochondrial membrane of heart muscle cells and parasites in experiments employing diaminobenzidine and tetrazolium salts, respectively. ATP synthetase complexes were found arranged along the tubular cristae in heart muscle cells, being visible as gold complexes in conventional microscopy and electron spectroscopic imaging (ESI). Applying the Oschman and Wall technique (1972) and ESI we could study the behaviour of mitochondrial profiles in mouse heart muscle cells, in *T. cruzi* and during the parasite cell cycle inside the cell, emphasizing with high contrast and resolution the differences between the parasite mitochondrion and the mammalian cell mitochondria. The present data showed that several aspects of the **in vivo** mouse muscle dynamics were reproducible in the **in vitro** system. The role of mitochondria in energy transport in muscle cells is discussed.

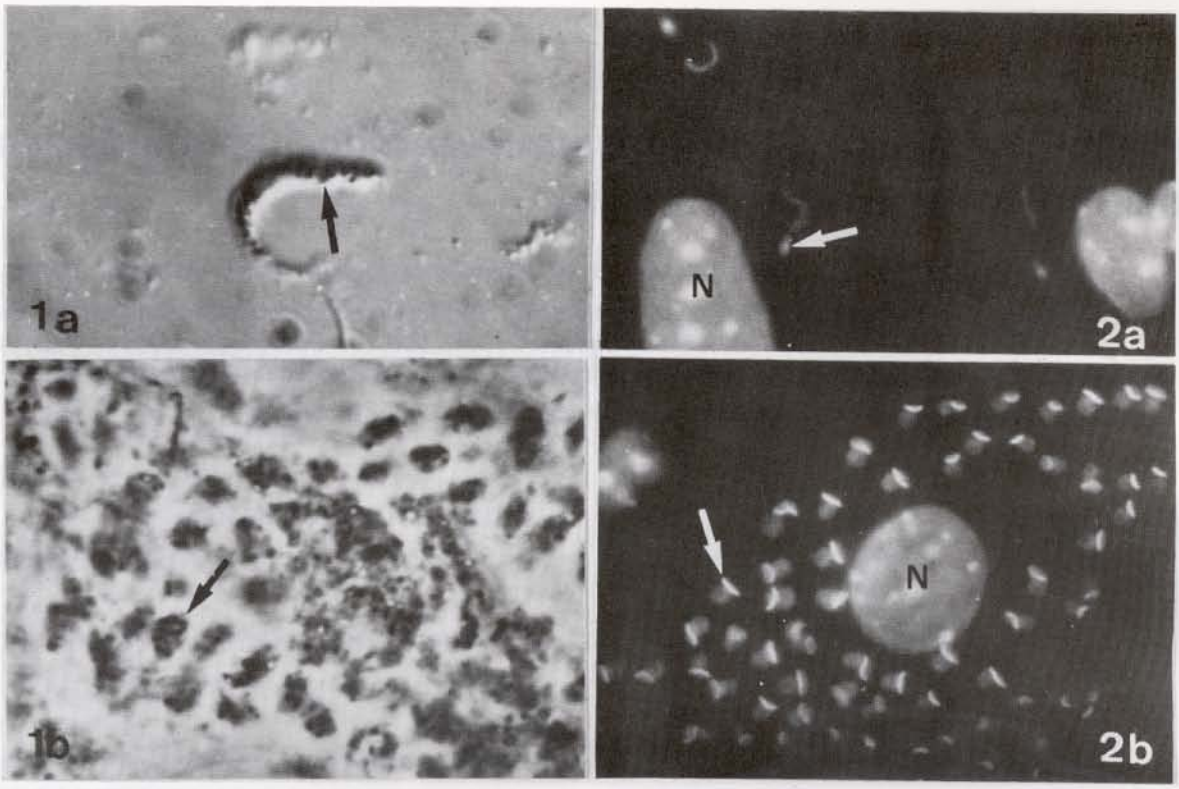
Key Words: Mitochondrial enzyme cytochemistry - Immunocytochemistry - Ca^{2+} binding sites - Mice heart muscle cells - **Trypanosoma cruzi** - Electron Spectroscopic Imaging (ESI).

Introductory Remarks

This paper reviews our work on mitochondria cytochemistry and imunocytochemistry and discusses the use of calcium cytochemistry in the context of relevant literature.

Mitochondria are highly pleomorphic organelles that exhibit a wide range of shapes (such as round,elongated, rod-like) and a number of dynamic features (such as fusing, dividing, branching and bending). Sites of electron transport, oxidative phosphorylation, tricarboxylic acid cycle reactions and several kinds of enzymes activity have been identified in the mitochondria. An important finding in the early 1960's was the discovery of the presence of DNA in organelle. DNA was reported in abnormal fibroblasts mitochondria, in the Trypanosomatidae protozoa, in yeast cells and in certain other cells (Clark and Wallace,1960; Steinert, 1960; Nass and Nass, 1962).

The cytochemical localization of enzymes involved in the oxidation-reduction cycle - in particular those embedded in the mitochondrial inner membrane that participate in electron transport - has been investigated in several studies (Seligman et al, 1968; 1971; Spector,1975; Meirelles and De Souza, 1982; Sokal et al, 1989). The activity of these enzymes has been found to vary in accordance with the metabolic state of cells (Halestrap, 1989).



Figs. 1a, 1b *- Normarski optics adjusted to amplitude contrast: a) reaction product of NADPH tetrazolium reductase activity was observed as granules in the body of a trypomastigote form of parasite; b) an infected HMC shows ICDH activity. Granules of NBT reaction within the amastigotes forms of the parasite and also on the host cell' cytoplasm were visualized (arrows). X 3,600; X 2,400.*
Figs. 2a, 2b *- DAPI staining of the the nucleus: a) three parasites were seen invading HMC and displayed an elongated nucleus and a rounded K-DNA; b) 3-days old HMC infected with T. cruzi. The intracellular parasite showed a bar-like kinetoplast (white arrows). X 1,400; X 1,400.*

Oxidative phosphorylation is the process through which energy released by oxidation reactions in the electron transfer chain is used for ATP synthesis. In mitochondria, ATP synthesis takes place on the inside surface of the inner membrane in a process that has been extensively studied (Cross, 1981; Tzagoloff,1982; Muller - Hocher et al, 1985; Allen et al,1989; Liveanu et al 1991; Corte-Real and Meirelles,1991).

We have been working with heart muscle cells from different sources (mouse embryos, chicken embryos and adult tissues) in our studies of host cell interactions with **Trypanosoma cruzi**, the causative agent of Chagas' disease (Chagas,1909). This disease - an endemic public health problem in Latin America - involves parasite- induced destruction of heart muscle, leading to heart failure, several types of cardiac block and arrythmia.

In our experimental system, we have used ultrastructural cytochemistry to characterize oxidation-reduction enzymes that participate in the electron transport or in ATP synthesis. Also we analysed events related with parasite invasion and multiplication within cells, on certain aspects of myogenesis, on the organization of cytoskeletal networks in normal cells and during parasite - host cell interaction (Meirelles and De Souza, 1982; Meirelles et al, 1986; Barbosa and Meirelles,1992; Pereira et al,1991).

Heart Muscle Cells and Trypanosoma cruzi

The life cycle of **Trypanosoma cruzi** involves differentiation through three distinct parasite forms : epimastigotes, which replicate in the insect vector, trypomastigotes which are infective and non replicative, and amastigotes which proliferate intracellularly in mammalian cells by binary fission. During the transformation of proliferative forms into infective forms, important morphogenetic changes are known to occur. One of the most striking of these modifications occurs at the level of the kinetoplast structure, a structure which in *T. cruzi* contains 15-20 per cent of total DNA, arranged as small circular molecules, known as minicircles and maxicircles, that encode several proteins and RNAs essential for electron transport and oxidative phosphorylation. K-DNA is aligned as a regular stack of fibers inside the mitochondrion at the base of the flagellum (Silver et al, 1986; Simpson,1972). In the amastigote and epimastigote proliferative forms of the parasite, the K-DNA must replicate before their transformation into the non replicative trypomastigote form. In this process, the K-DNA changes from a compact, bar-like form to a looser, basket-like structure (Meyer, 1968; Silver et al,1986). Confirmation of the unitary nature of the *T. cruzi* mitochondria was obtained with

reconstructions based on serial thick sections and stereoscopic photography. The functional significance of a unitary mitochondrion may be related to equal distribution of mitochondrial elements during cell division (Paulin, 1975).

The primary cultures of heart muscle cells (HMC) were prepared by taking the hearts of 18-day old mouse embryos, mincing them and incubating for 10 min. at 37°C in 0.05% trypsin and 0.01% collagenase (Worthington) in a Ca++Mg++ free PBS solution, as already described (Meirelles et al,1986). The cells were plated on a gelatin-coated substrate and underwent the myogenic process. We also worked with heart muscle prepared from chick embryos and cultivated in a plasma clot using a mixture of Tyrode solution, inactivated serum and chick embryo extract for the liquid supernatant phase (Meirelles and De

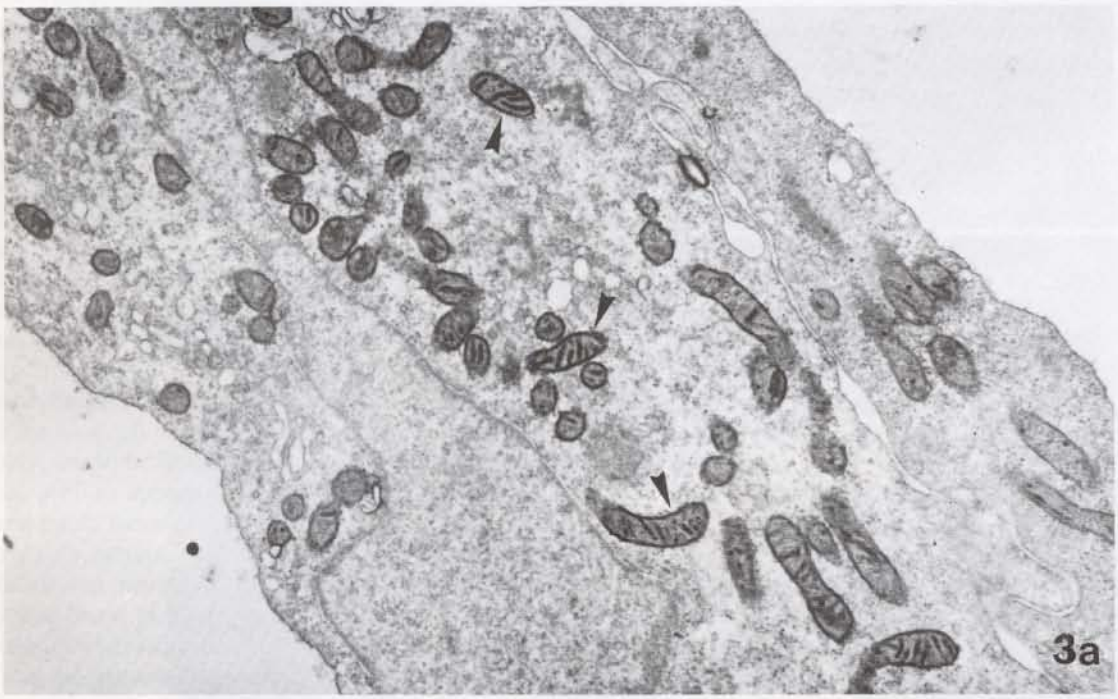


Fig. 3a) *- HMC displaying numerous mitochondrial profiles with an strong activity of cytochrome oxidase at the internal membranes.X18,000;*

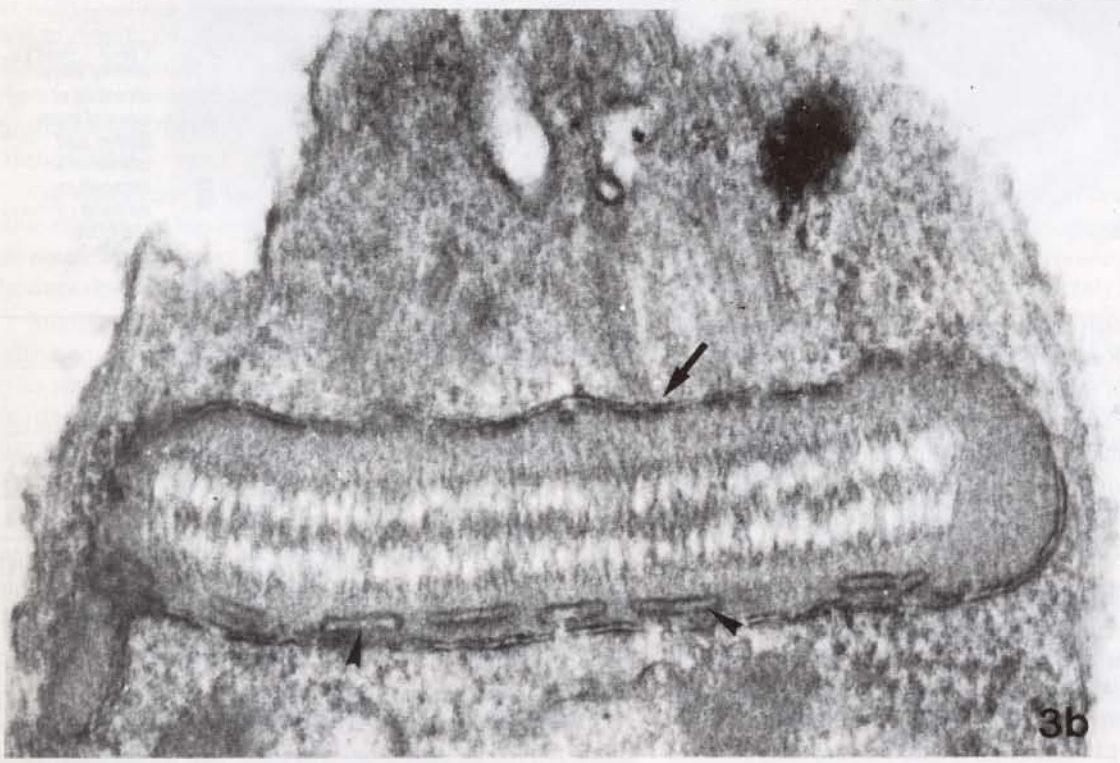


Fig. 3b) *- Intracellular forms of T. cruzi showed cytochrome oxidase activity in the internal cristae of the single mitochondria.X 95,000.*

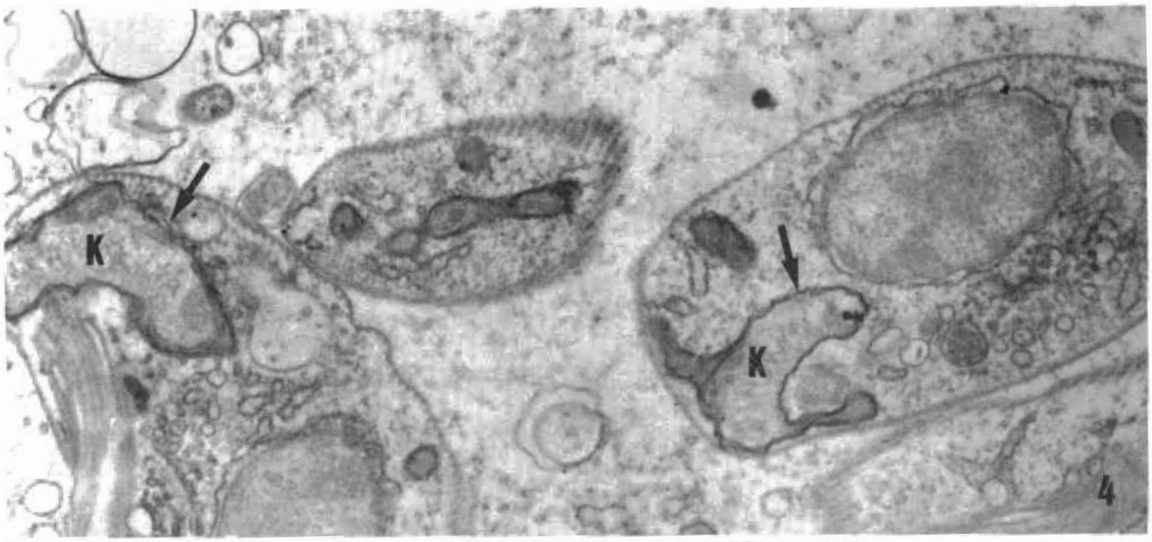


Fig. 4 - Ultrastructural localization of NADH tetrazolium reductase showing the reaction product in the mitochondrial inner membrane of the parasites that were free in the cell's cytoplasm (arrows). X 22,000.

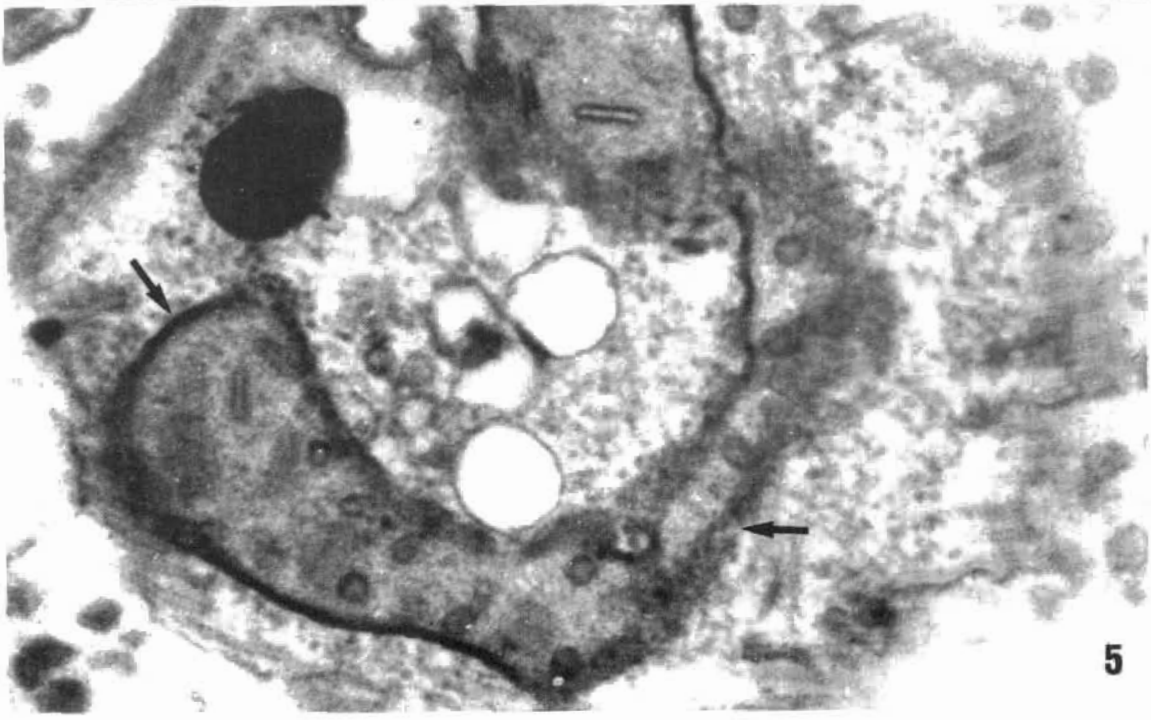


Fig. 5 - ICDH activity were localized at the internal membrane and cristae of an intracellular parasite (arrows). X 34,000.

Souza,1982). Finally intact HMC from adult mice were also used (Corte-Real and Meirelles,1991). For studies of parasite - host cell interactions we used *T. cruzi* Y strain isolated from an acute human case of Chagas' disease. Bloodstream forms of the parasite were obtained from infected mice at the peak of parasitemia, after 7 days of infection. The parasites were isolated by differential centrifugation (Meirelles and De Souza, 1984a).

Our experimental protocols used monolayer cultures of HMC from chicken or mouse embryos and intact cells from adult mice. Infection was achieved by incubating cells in the presence of parasites for 24 to 96 hours, subsequently they were rinsed and prepared for cytochemical and immunocytochemical investigation of mitochondrial enzymes.

Mitochondrial Enzyme Cytochemistry

For the cytochemical detection of oxidation-reduction enzymes that participate in electron transport - we followed previously established protocols (Pearse,1968; Seligman et al, 1971; Spector,1975). When attempting to localize these enzymes, we had to balance the need for structural preservation against the requirements to maintain the enzyme activity. Our best results were obtained when the cells were fixed in a solution containing 1% of paraformaldehyde and 1% glutaraldehyde.

For the detection of cytochrome oxidase, diaminobenzidine (DAB) methodology was used; and for the localiza

tion of the dehydrogenases activity we employed two tetrazolium salts, osmiophylic dystiryl nitroblue tetrazolium (DS-NBT) and nitroblue tetrazolium (NBT)-active electron acceptors, respectively for the electron and light microscopic observations (Meirelles and De Souza,1982).

The following dehydrogenases were localized in normal and *T. cruzi* infected HMC, in intracellular parasites and in parasites released in the culture medium after undergoing the intracellular stage of the life cycle: succinate dehydrogenase (SDH), isocitrate dehydrogenase (ICDH), ß hydroxybutyrate dehydrogenase (ß HBDH), glycerophosphate dehydrogenase (GDPH) and nicotine adenine dinucleotide phosphate tetrazolium reductase (NADPH).

Under the light microscope, with Normarski optics adjusted to amplitude contrast, the enzymes involved in electron transport pathway could be detected by reference to the blue granules that formed as a result of the nitroblue tetrazolium reaction. These granules filled the host cell cytoplasm and were particularly evident around the nucleus and within intracellular parasites. Inside the parasites, granules were seen scattered thoughout the parasite body, with the number of granules varying from 5 to 15 (Fig 1a, 1b). DAPI (4',6'- diamidinophenylindole) staining of DNA showed that in addition to DNA in the host cell and in parasite nucleus, a further quantity of DNA was to be found inside parasite, with rounded and bar-like profiles appearing, respectively, in trypomastigote and amastigote forms (Figs. 2a, 2b). At the electron microscopic level, cytochrome oxidase could be detected both in HMC - on the internal membrane and in the cristae - and in all developmental forms of the parasite (Figs. 3a, 3b). Dehydrogenases were found associated with the inner mitochondrial membrane, along the cristae with varying levels of intensity from moderate for SDH to strong for NADPH and ICDH. Our group was the first to report the presence of the ICDH, ß HBDH, GPDH (NAD-not linked) in *T. cruzi* (Meirelles and De Souza, 1982). Fig. 4 and 5 show certain aspects of the distribution of NADPH diaphorase and ICDH in the mitochondrion of intracellular *T. cruzi* in HMC.

Our conclusion is that host cells and parasites possess the same enzymes of the oxidation-reduction cycle and that there are not clear differences in the respiratory metabolism among the three developmental forms of the *T. cruzi*.

Studies currently underway in our laboratory on the localization of mitochondrial enzymes in amphibian reticulocytes suggest that modifications in the expression of the oxidation-reduction enzymes occur at different stages of the maturation process of these cells (Cianciarullo and Meirelles,1991).

The ATP Synthetase Complex

The mitochondrial ATP synthetase complex is bound to the inner mitochondrial membrane and catalyses the formation of ATP, the latter being coupled to H+ translocation across the membrane. For the localization of F1 ATPase in intact HMC of adult mouse we carried out immunocytochemistry on cryosections of unembedded tissue as described elsewhere (Tokuyasu,1973; 1980; Griffiths et al, 1980;1984).

Ventricles from mouse heart muscle were cut into 1mm pieces and fixed in 4% PFA,then immersed in 2.3 M sucrose in 0.1M Ca++ and Mg++ free phosphate buffered saline, as previously described (Geuze and Slot, 1972). Frozen sections were obtained in a RMC cryoultramicrotome. Antibody labeling was carried out using the method of Griffiths et al (1980).

Working with intact heart muscle tissue, we observed mitochondria as branching systems located above and below Z lines and spreading across the entire diameter of the muscle fiber. We localized F1 gold complexes along the tubular cristae of the HMC mitochondria that were arranged in parallel to Z lines at distances corresponding to the muscle fiber diameter. We applied the electron spectroscopic imaging technique (ESI) allowing selective contrast enhancement and improvement in image quality (Ottensmeyer et al, 1980; Bauer et al, 1985). The gold particles were clearly resolved in the mitochondrial cristae, confirming the arrangement of F1 factor in the mitochondrial inner membrane (Fig. 6a), as described by Allen et al (1989) in the mitochondria of Paramecium using rapid-freeze deep-etch techniques.

As already described (Bakeeva et al, 1978; Bakeeva, 1983) mitochondria are coupled to each other by specific intermitochondrial contacts and they are organized as a network spanning the I band regions of the muscle near the Z-lines. These contacts contribute to the unification of the mitochondrial system within the cell, thus facilitating the transport of the energy in the form of electrical potential (Skulachev,1980). Cryosections of adult mouse HMC, as described here, confirm the organization of this mitochondrial network, showing rows of mitochondria running along and integrated with the myofibrils (Fig. 6b).

The HMC Mitochondria And Its Integration Within The Cells: Calcium Binding Sites

Calcium ions are known to occur in three differents forms in the living cells: the free cellular calcium that is lost with other soluble cations during tissue processing for electromicroscopy; the Ca++ that is tightly bound in calcified tissue; and the remaining calcium that exists in subcellular reservoirs and represents the exchangeable Ca++ that can be detected using histochemical methods (Wick and Hepler, 1982; Probst, 1986). Ionic calcium play an essential role in many biological phenomema, such as muscular excitation-relaxation cycle, membrane processes, flagellar and ciliary activity, etc. as described in several reviews (Martonosi,1989; Carafoli and Penniston,1985; Jacobson et al,1985; Rassmussen and Barret,1984).

Calcium also has been implicated in several dynamic processes of mitochondria within cells, such as the organization of the inner membrane and its matrix compartment, and fusion and fission events (Bereiter-Hahn, 1990). Enzymes of the citric acid cycle may be regulated by changes in mitochondrial Ca++ concentration in situ (Coll et al, 1982).

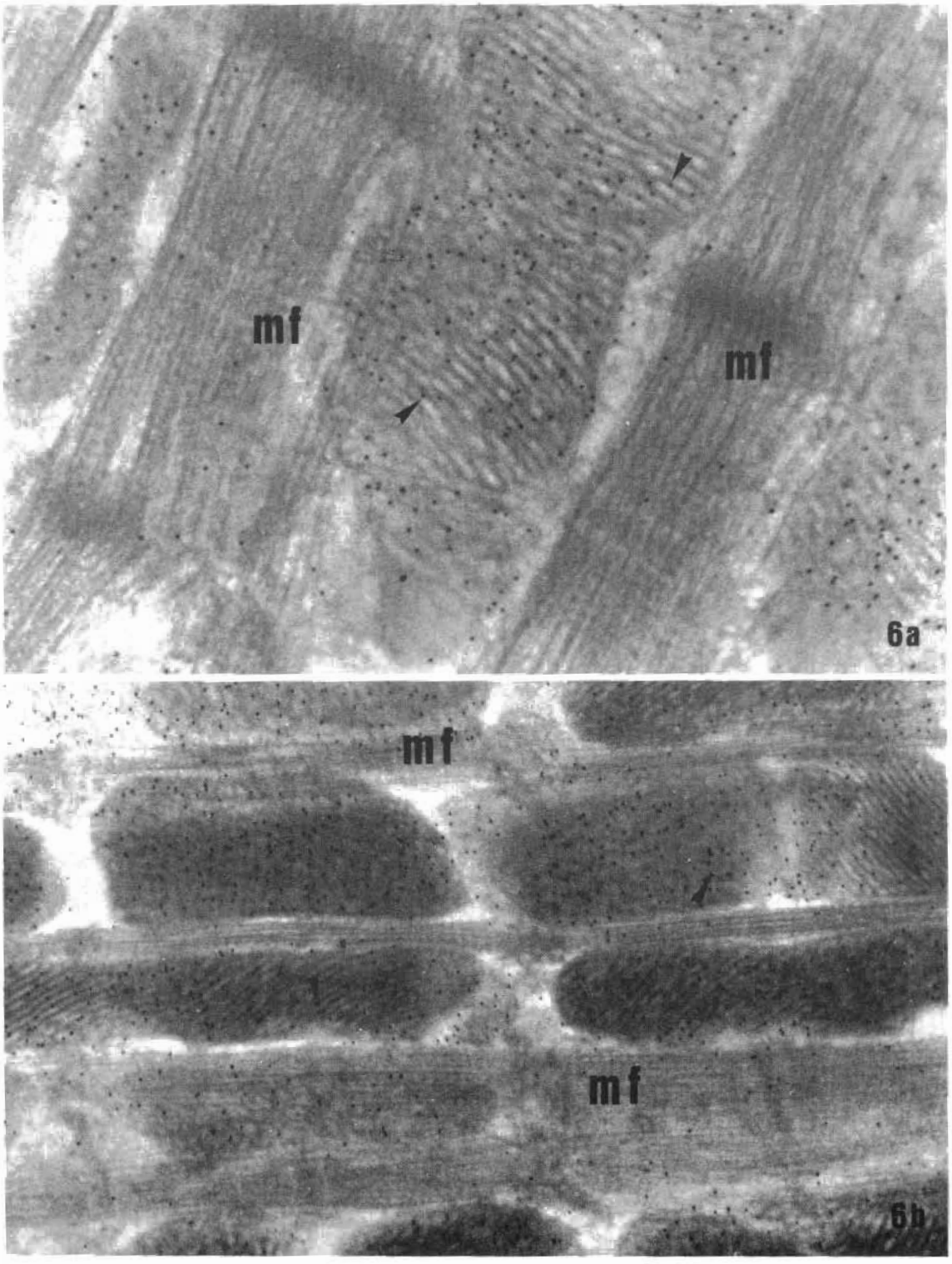


***Fig. 6a, b** - 6a) Electron spectroscopic imaging (ESI) with an energy loss of 111 eV displayed F1 ATPase as gold complexes associated to inner mitochondrial membranes on cryosections of adult HMC (arrowhead); b) General view of cryosections of adult muscle cells showing an organized array of mitochondria and myofibrils (mf). The inner mitochondrial membranes were labeled with coloidal gold particles indicative of the presence of F1 ATPase (arrowhead). X 72,000; X 45,000.*

It is widely accepted that mitochondria isolated from most tissues of vertebrates, as well as from lower life forms, possess an active Ca++ transport system believed to participate in intracellular calcium homeostasis (Do Campo and Vercesi,1989).

When Ca++ enters mitochondria via the electrogenic uniport, the membrane potencial decreases and pH increases. If phosphate is present, it will enter in response to the increased pH, restoring the pH and the inside negative membrane potential to their previous value. Calcium phosphate precipitates inside the mitochondrial matrix, leading to very extensive Ca++ accumulation. When cells are injured they take in large amounts of calcium from the extracellular medium and mitochondria are able to absorb the excess thanks to their buffering ability. But if

excessive amounts of calcium are taken in, overcoming mitochondrial storage capacity, many additional reactions are activated, leading to cell death. Mitochondria also display matrix granules that can discerned from the electron dense particles that have a large content of calcium. It has been described that in heart muscle cells in stimulating situations matrix granules appear to create contact between inner and outer mitochondrial membranes in which enzymes can function efficiently (Jacob et al., 1994).

For the localization Ca++ binding sites, we applyed the Oschman and Wall technique (1972), that consists in immersing cells in 5mM CaCl2 together with the glutaraldehyde fixative (GA) in 0.1M Na cacodylate buffer or 0.08M s-collidine buffer, pH 7.2, plus 3.5% sucrose. Subsequently, cells are post-fixed in 1% OsO4 plus 2.5mM CaCl2. We also used other concentrations of CaCl2 in the first fixative in the range of 10 - 20 mM. Modification of this protocol included the addition of 0.8% potassium ferrocyanide during the post-fixation step. As a control, the cells were fixed without CaCl in GA.

When calcium is added in all solutions during the fixation of the cells, a remarkable enhancement of some membranes is visualized. According Oschman and Wall, a significant part of the density might be due to elements originally present in the cells and the effect of the calcium might be to stabilize componentes of the cells. The authors discuss that the images are suggestive that the calcium-binding component is closely associated with plasma membrane, if not a component of the membrane itself. In the samples treated with osmium and ferrocyanide a brown precipitate formed, due probably to the oxidation of ferro to ferrycyanide with concomitant reduction of osmium.

Most of the **in vitro** studies of heart muscle cells have been done using rats and chick embryos, only few ones using mouse embryos. Ultrastructural studies of mouse myocardium **in vivo** adding calcium or other divalent or trivalent cations to the fixatives have shown with high enhancement a number of features that ordinarily would go unnoticed (Forbes et al,1977).

We established an experimental system of heart muscle cells from embryogenic mouse suitable for morphological, kinetics and cell diferentiation studies (Meirelles et al, 1984b;1986). During myogenesis **in vitro**, heart muscle cells - after an initial period of cell proliferation - follow the normal myogenic process, with sequential cell coupling and fiber formation. At this stage it is often common to find the coupled cells presenting specialized membrane regions, such as desmosomes and gap junctions, with the result that each cell may display a different mitochondrial profile.

With both the Oschman and Wall technique and the electron spectroscopic imaging (ESI) we could study the the behaviour of mitochondrial profiles in mouse HMC, in *T. cruzi* and during the parasite cell cycle inside the cells. ESI allows the imaging of thick and ultra-thin unstained specimens, producing sharp results in respect of both contrast and resolution, irrespective of specimen thickness (Bauer,1988). Thick specimens (such as the ones we used) can be observed if contrast tuning is performed by selecting an energy window of inelastically scattered electrons.

We found a giant mitochondrion traversing the longitudinal axis of the HMC and displaying a high electron density that suggested the possible presence of calcium binding components associated to its membrane. Other mitochondrial profiles could be seen in the same cell, and signs of mitochondria fusion or fission were observed. We could see arrangements of contractile proteins in sarcomeric units (Fig. 7).

Fig. 7 - HMC that were incubated with 5mM CaCl2, in two steps of the fixation, sho-wed a giant mitochondrion lying within cell' cytoplasm with a high electron density in its membranes. Other mitochondrial profiles were observed in the vicinity. Signs of fusion or fission were seen (two arrowhead). X 30,000.

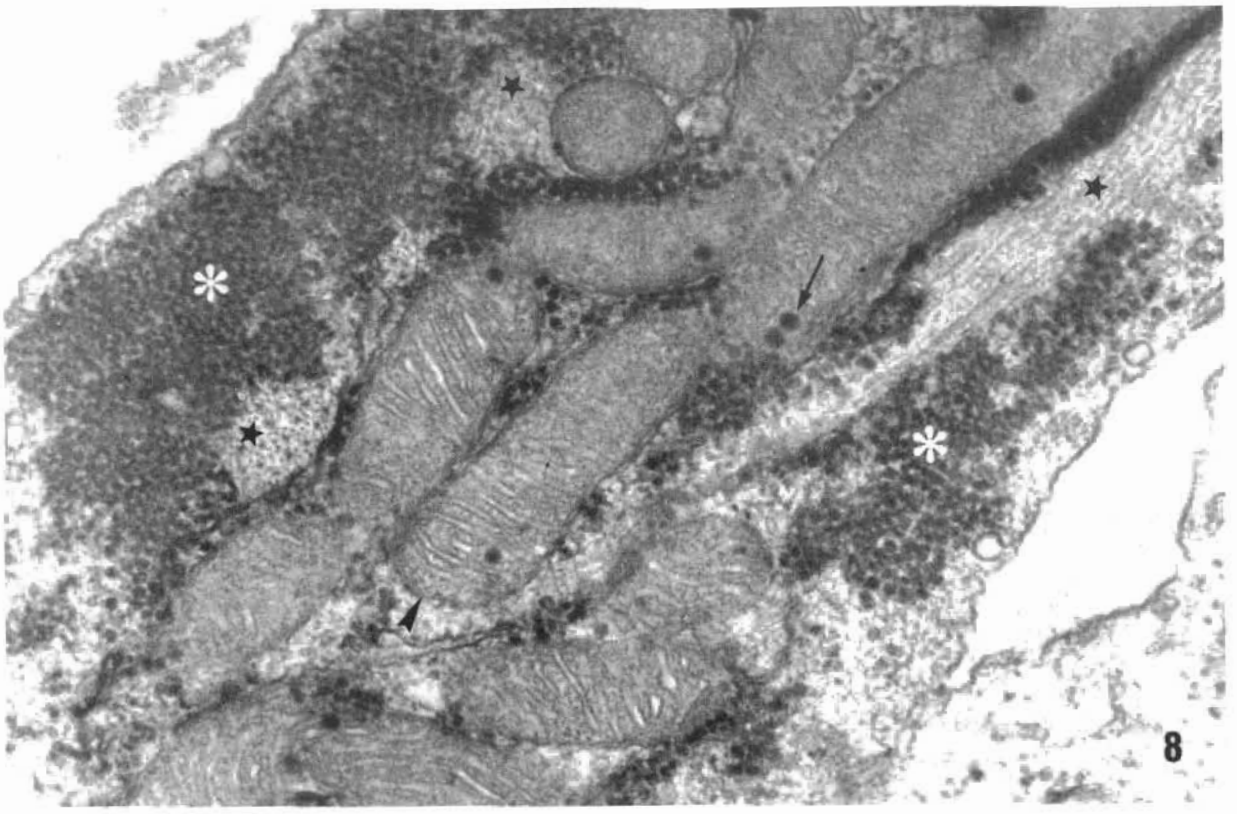
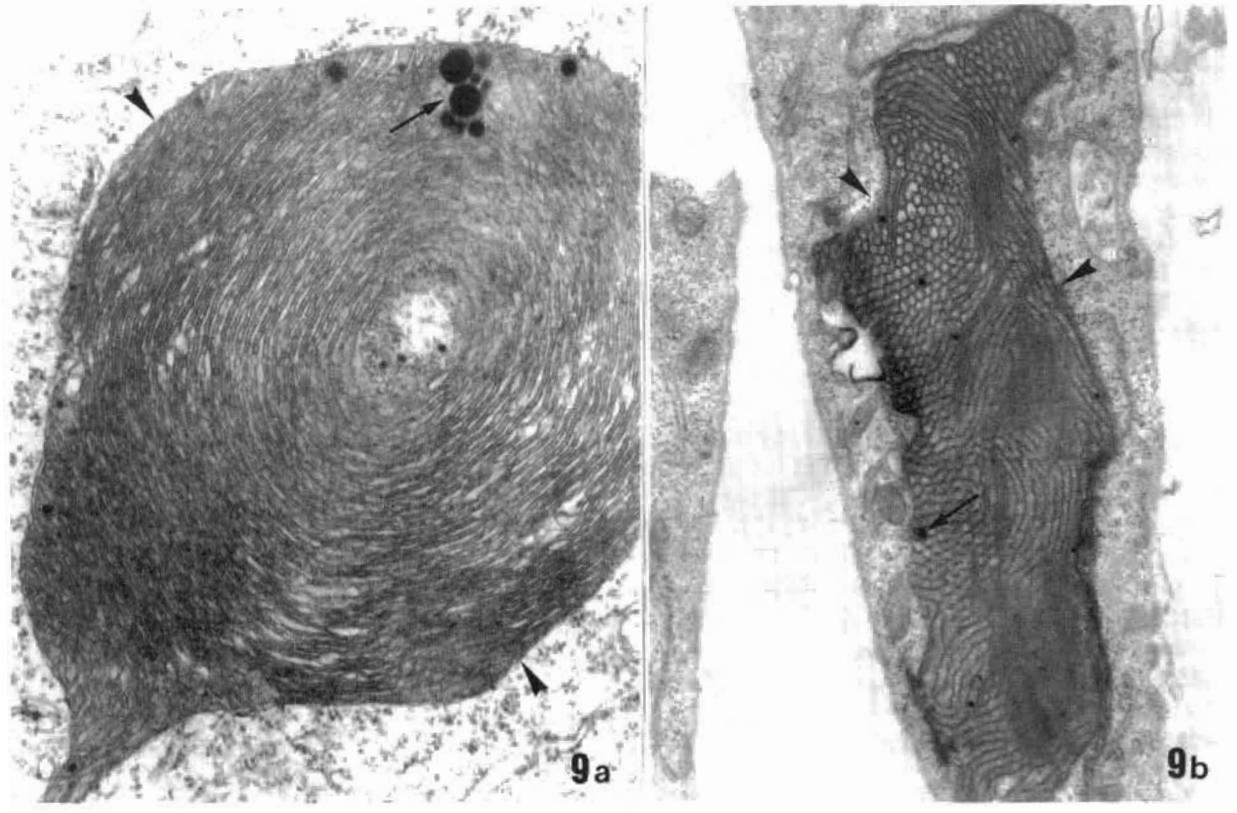


Fig. 8 - Myofibrils in transversal and longitudinal sections ( ) surrounded by packed mitochondria exhibited calcium accumulation in the form of hydroxyapatite crystals (thin arrow). Clusters of glycogen ( ) were observed in the cell'cytoplasm. X 88,830.



Figs. 9a ,9b - HMC incubated with 20mM CaCl2 presented some unusual mitochondrial profiles with different arrays of internal membranes, showing enhanced contrast. X 41,600; X 29,700.

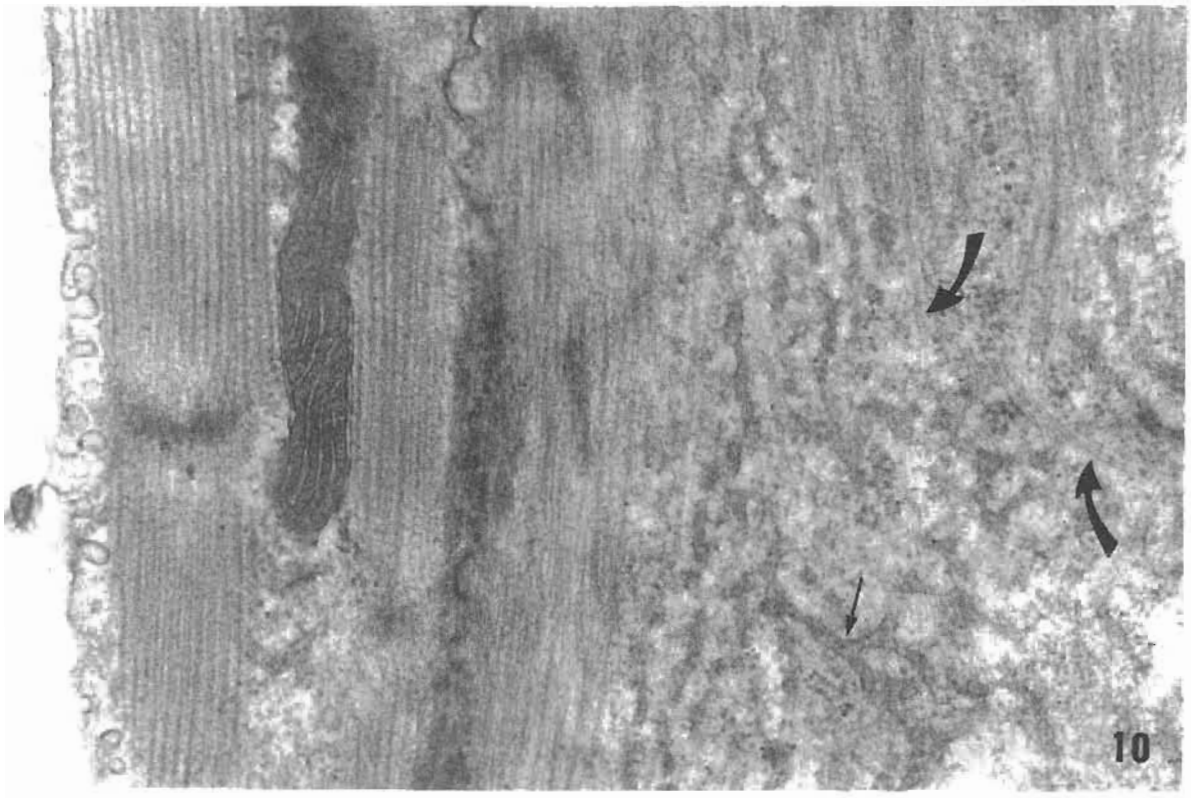


Fig. 10 - General wiew of 7-day old HMC. Numerous caveolae were localized at the sarcolemmal membrane and organized arrays of myofibrils and mitochondria were displayed. A developed sarcoplasmic reticulum (thin arrow) was observed in close association with microtubules (curved arrows). X 65,000.

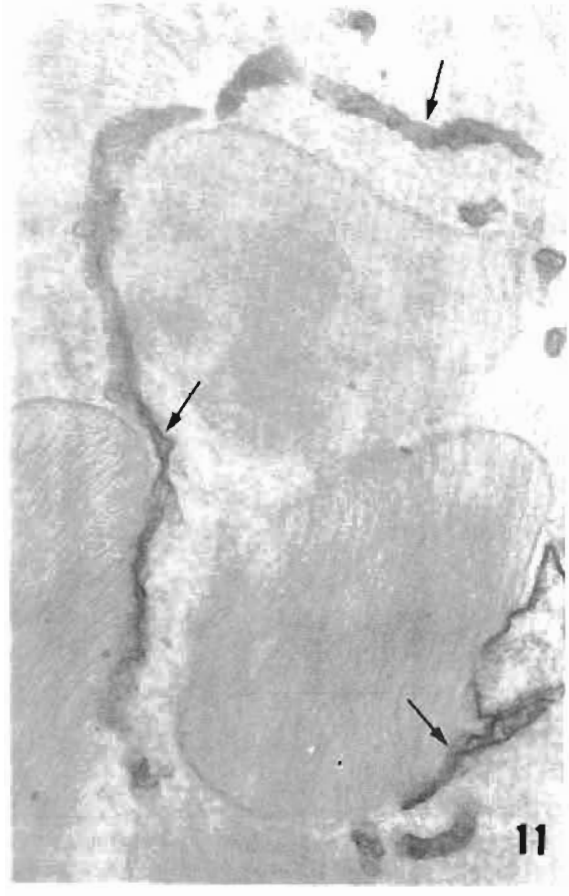


Fig. 11 - HMC incubated with 5mM CaCl2 and ferrocyanide complex revealed enhanced sarcoplasmic reticulum (thin arrow) encircling mitochondria. X61,000

Fig. 12 - Mitochondrial-microtubule association was observed in the cell' cytoplasm of HMC (arrows). X 65,800

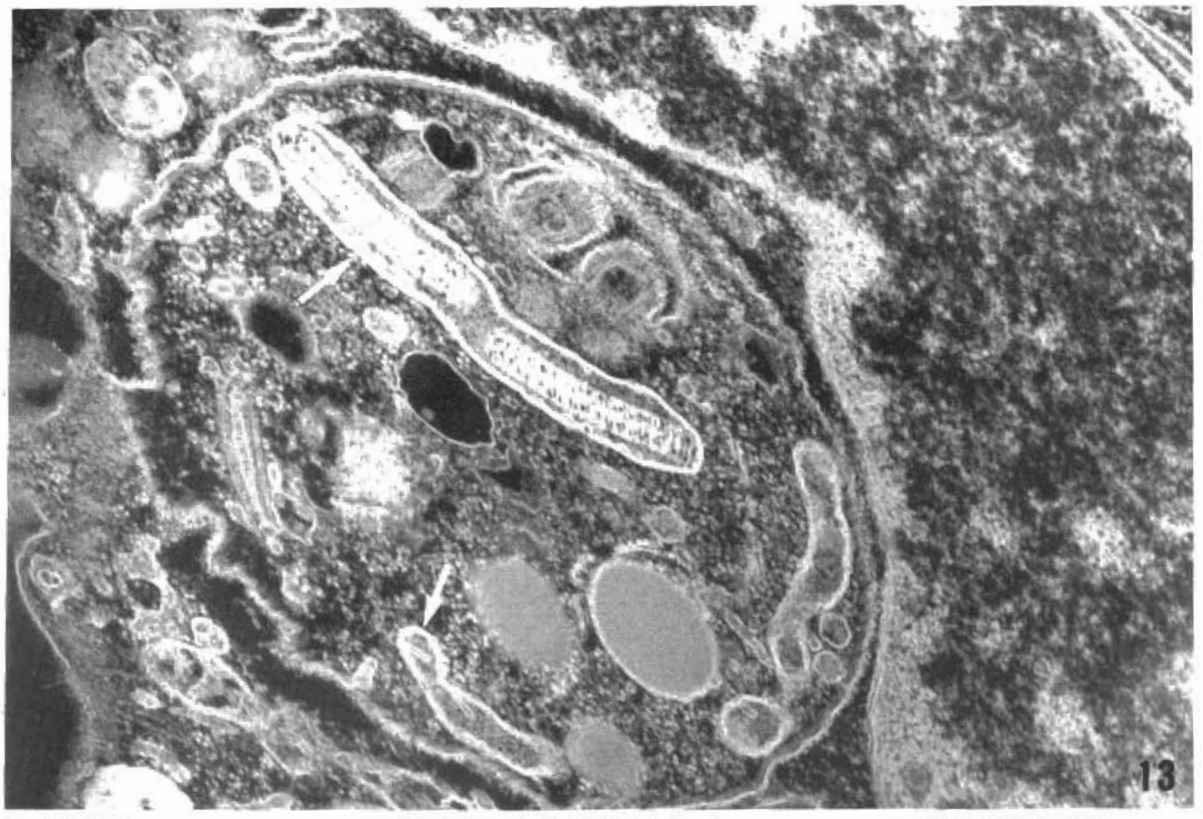


Fig. 13 - ESI imaging of T. cruzi infected HMC treated with CaCl2, in both fixation steps, showed with high contrast the inner mitochondrial membrane of the intracellular parasite with an energy loss of 215 eV. (white arrows) X 36,000.

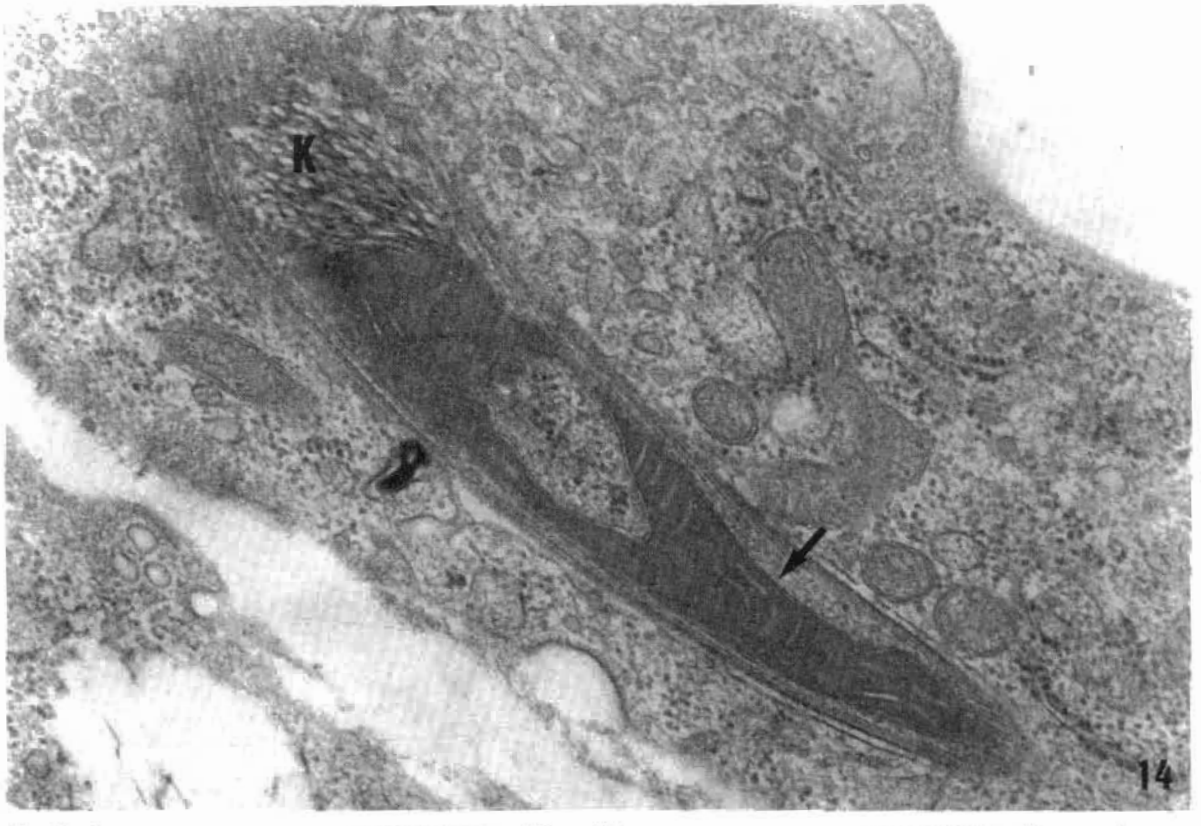


Fig. 14 - Conventional transmission electron microscopy of T. cruzi infected HMC, showing a trypomastigote form of the parasite, immediately after the invasion of the cell, inside an endocytic vacuole. This infective form displayed a basket-like form of K-DNA inside a well developed mitochondrion (arrow). X 65,000.

contracting HMC, treated with Ca++ in both fixatives and with 0.8% potassium ferrocyanide, we localized transverse and longitudinal profiles of myofibrils surrounded by packed mitochondria with prominent Ca++ crystals in their matrix. In the cell cytoplasm there were abundant clusters of glycogen (Fig. 8), probably used for generation of ATP molecules. It was also observed cells with enlarged mitochondria with unusual arrangements of the inner membrane (Fig. 9a; 9b), and along with conventional profiles of mitochondria.

The organization of cells grown in culture for 7 days, displayed HMC with numerous caveolar clusters located at the surface of the sarcolemma and in the cytoplasm. Sometimes the caveolae formed beaded tubules suggestive of possible generation of new T tubule (TT) (data not shown). Mitochondria were observed in clefts between myofibrils.The sarcoplasmic reticulum was visible as an extensive network exhibiting enhanced profiles, and showing close association with microtubules (Fig.10).

In cells treated with ferrocyanide and osmium an enhanced sarcoplasmic reticulum was seen encircling mitochondria (Fig. 11). In some cells clear association of microtubules with mitochondria was observed (Fig. 12).

During the cell cycle of the intracellular form of the parasite within HMC different arrangements of the K-DNA inside the mitochondrion was visualized. Electron spectroscopic image obtained by appropriate selection of inelastic electrons with an energy loss of 215 eV, gave high levels of contrast and details revealing the intracellular parasite with a strong enhancement of their mitochondrial membranes. The K-DNA in the process of division was visible as a bar-like shape inside this mitochondrion (Fig. 13). The infective form of the parasite - the trypomastigote form - displayed K-DNA as a basket-like structure inside well-developed, cristate mitochondria, immediately after invasion of HMC and while inside the endocytic vacuole (Fig. 14).

Conclusions

The mitochondria in muscle cells are energy-producing organelles, coupled to each other by numerous specific intermitochondrial contacts. They are morphologically dynamic and display remarkable plasticity. It is well accepted that mitochondria play a vital role in energy metabolism, and that they are essential for the survival and proliferation of cells.The present data showed that several aspects of mouse muscle dynamics **in vivo** are reproducible in the **in vitro** system. The study of mitochondria in mouse heart muscle cells and during its interaction with **Trypanosoma cruzi** could prove to be one of the most interesting approaches for elucidating intracellular energy transport mechanisms and for investigating how energy metabolism and energy requirements are channeled into different functions. Our system using primary cultures of mouse heart muscle cells facilitates the control and modification of cell culture. Employing this system for the purposes of molecular, biochemical and ultrastructural analysis offers an excellent experimental framework for the study of mitochondrial interactions within cells.

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