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FURTHER STUDIES ON THE FINE STRUCTURE OF THE KINETOPLAST-CHONDRIOME OF *TRYPANOSOMA (SCHIZOTRYPANUM) CRUZI* IN THIN SECTIONS OF INFECTED TISSUE CULTURES

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SUMMARY

The fine structure of *Trypanosoma cruzi* in tissue cultures has been examined, in order to study the changes in the kinetoplast-chondriome which occur during the various phases of the life cycle of the parasite, and to learn about the significance of a new structure, observed recently in the kinetoplast of some extracellular adult forms.

It has been verified that both of the structures observed, the fibrous one, which is usually described, and the basket-like structure discovered more recently, are of trypanosomes which are able to enter a tissue cell and begin a new cycle.

In the beginning of a new cycle, when the spherical kinetoplast is transformed into a more rectangular or lentiform structure and migrates to the anterior half of the body, the basket-like structure can still be recognized. It is, however, no longer found, when many leishmania bodies are contained in a cell. The kinetoplast then shows the dense lamellar mass of great electron density, usually described in this form.

In some leishmania bodies a central, horizontal line has been observed in the kinetoplast, which is supposed to represent the last step of the reduction of the basket-like structure, seen in the trypanosome form.

Also in the adult form, at the end of the intracellular cycle, when the trypanosomes are still confined in the host cell, this structure has not been encountered, and it is supposed, therefore, to represent the final point of the life cycle up to which the parasite may develop, but must not necessarily do so, in order to begin a new cycle.

The real nature of this new structure continues to be uncertain, and its possible significance is being discussed.

INTRODUCTION

In a previous paper [2] some structures were described in *Trypanosoma cruzi*, maintained in tissue cultures, which had not been observed before [4, 5]. These structures appeared especially in the flagellum and in the kinetoplast of the trypanosome, after double fixation with glutaraldehyde and osmium tetroxide and embedding in Epon. In the present paper, further observations will be given concerning the kinetoplast only.

In the electron micrographs of the kinetoplast, after fixation with osmium tetroxide only and embedding in methacrylate, it was found that it is a vacuole-like, apparently lentiform structure, which is separated from the cytoplasm by a double membrane [4, 5].

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It contains, in the leishmania forms, a lamellar mass of great electron density, in which the lamellae are disposed in parallel array, in the direction of the body's length axis. It was seen in favorable sections that the whole vacuole formation is continuous with the mitochondria of the leishmania body. Mitochondrial cristae were also found at its periphery [12].

In the trypanosome form, at the end of the intracellular cycle, the kinetoplast appeared less electron dense. The lamellar mass was seen to be transformed into long fibres of irregular shape, which were more dispersed than in the leishmania bodies. They still showed the parallel array and orientation in the direction of the body's length axis [5]. However, because of the intense undulating and contracting movements of this form *"in vivo"*, at the moment of fixation it had not been possible to obtain sufficiently complete images of this region of the trypanosome in thin sections, so that the continuity of the kinetoplast with the mitochondria and the other structures of the body was uncertain.

With improved methods, however, (embedding in Epon) better preparations were obtained, and it was seen that also in the trypanosome form, at the end of the intracellular cycle, the kinetoplast is continuous with the mitochondria [2]. It is found in an enlarged region of a long mitochondrion which begins near the nucleus and reaches up almost to the posterior tip of the parasite. This was equally seen in the free, extracellular trypanosomes of the cultures which had left the host cells after the end of the cycle.

Among these free extracellular trypanosomes, some parasites were found with a structure which had never been observed before. In these, the kinetoplast had a basket-like structure which was composed of small units of rectangular or fusiform shape, quite similar to mitochondrial cristae, disposed orderly in horizontal lines, the tips oriented in the direction of the body's axis. Here also, continuity with the mitochondria was visible. Since only very few of these forms had been found at that time, it was not possible to explain the significance of this structure in the life cycle of the parasite. Several explanations seemed possible, one of them was that it is a degenerating form, another that it represented the kinetoplast of a metacyclic trypanosome, e.g. of a trypanosome at the final point of its life cycle, from where it would have to start a new cycle, penetrate a new host, or degenerate. A third possibility was that the two existing structures in the kinetoplast belong to parasites which are physiologically different, in which case even a sexual dimorphism might be considered.

In order to find out more details about the existence and the destiny of these forms in the tissue cultures, additional cultures were fixed and embedded and observed with the electron microscope.

MATERIAL AND TECHNIQUES

As in the previous work [2, 4, 5], whole hanging drop cultures, maintained in a plasma clot (*), were fixed which *"in vivo"*, under the optical microscope, showed a heavy infection with trypanosomes in all the forms of the cycle (**). The cultures were fixed with 2.5% glutaraldehyde for 30 minutes, and post-fixed with 1% osmium tetroxide for 1 hour, in the refrigerator. For dilution of both fixatives Tyrode solution was used, the same which had been employed for the culturing of the tissue cells, a method which had given good results in the study of tissue culture cells [7, 8]. Both fixatives were buffered to a pH of about 6.8 — 7.2.

Dehydration was done in acetone. Only after one night in 90% or pure acetone the interesting regions of the culture were cut out with a cataract knife and the dehydration of the small pieces was continued. They were then stained with uranyl acetate (0.3% in pure acetone) during one night. After repeated washings in pure acetone they were embedded in Epon Resine Shell.

(*) For tissue culture techniques see 3

(**) The trypanosome strain used came initially from human blood from the State of Goiás (Brazil), and has been maintained for more than 12 years now in tissue cultures and in mice

The thin sections were obtained with a PORTER & BLUM [9] microtome. They were stained with lead citrate [10]. The observations were done with a Philips electron microscope EM 100.

RESULTS AND DISCUSSION

Our attention was focused first on cells in which only one parasite was found, which apparently had entered the cell recently (Figs. 2, 3, 4, 5, 7). In such cells the parasites were on their way to assume the shape of the leishmania body, showing the kinetoplast at the level of the nucleus, or already in the anterior part of the body. Its large spherical shape appeared more rectangular. The orderly horizontal structure which had been observed in the kinetoplast of some of the free trypanosomes (Fig. 1), was also found in many of these forms (Figs. 3, 4, 7). Its continuity with the mitochondria was very clear in most of them.

In a more advanced state of the cycle, when more than one leishmania body was present in a cell, this structure was no longer found. All the leishmania bodies in such cells showed a kinetoplast with the dense osmiophilic structure, usually described in this form (Fig. 8). In some of them, however, a central horizontal line was seen which seemed to divide the lamellar mass in two rows (Figs. 9, 10).

In cells filled with the adult form of the parasite at the end of the intracellular cycle, the regular, horizontal structure, seen in the kinetoplast of the free trypanosomes, has not been found. All the kinetoplasts in such intracellular trypanosomes observed so far, showed the long, irregularly shaped fibres, but no horizontal structuration. In cells in which the transformation from leishmania to trypanosome was not complete, transition forms have occasionally been observed, in which one side of the kinetoplast was still in the form typical for the leishmania body, and the other already extended and showing the irregularly shaped fibres of the adult trypanosome (Fig. 11).

The results which have been obtained during these investigations show that the free, extracellular trypanosomes, in which the kinetoplast has the basket-like structure, composed of small units, arranged in horizontal lines, are no degenerating forms, but are, like the ones with the usual fibrous structure, able to penetrate a cell and to initiate a new cycle. The fact that this regular structure has not been found at the end of the cycle, when the trypanosomes were still confined in the host cell, suggests that it is the structure of a kinetoplast in trypanosomes at the final point of the cycle, a status which they probably seldom or never achieve, when still in the host cell, because this ruptures earlier, but which they may assume after the rupture of the host cell, in the interval between the end of the old and the beginning of the new cycle. This hypothesis is reinforced by the existence of extracellular forms, which have been found in the cultures, in which a horizontal structuration is just slightly traced, and which might represent transition forms (Fig. 6).

Nothing definite can be said so far about the nature and the significance of the regular structure. Considering the still uncertain role of the kinetoplast in trypanosomes in general, and in *Trypanosoma cruzi* specifically, the forms with the structure found recently by us deserve a very thorough investigation. The general opinion tends to attribute to the kinetoplast the role of mitochondria formation, which would be controlled by DNA found in rather large amounts in its fibrous material [1]. As stated before [2], it seems possible to us that in those forms with the basket-like structure, in which the irregularly shaped fibres have disappeared, all DNA is exausted by the prolonged extracellular stay of the parasite, and mitochondria formation has come to an end. During this extracellular stay the parasite is unable to synthesize new material and has to enter a new host, and begin a new cycle in order to survive.

It is not the aim of this paper to explain the reason for the increase of the mitochondria in the trypanosome form at the end of the parasite's life cycle. As VICKERMAN [14] and others suggest, this would be due to altered respiration conditions in the extracellular medium, which require a greater

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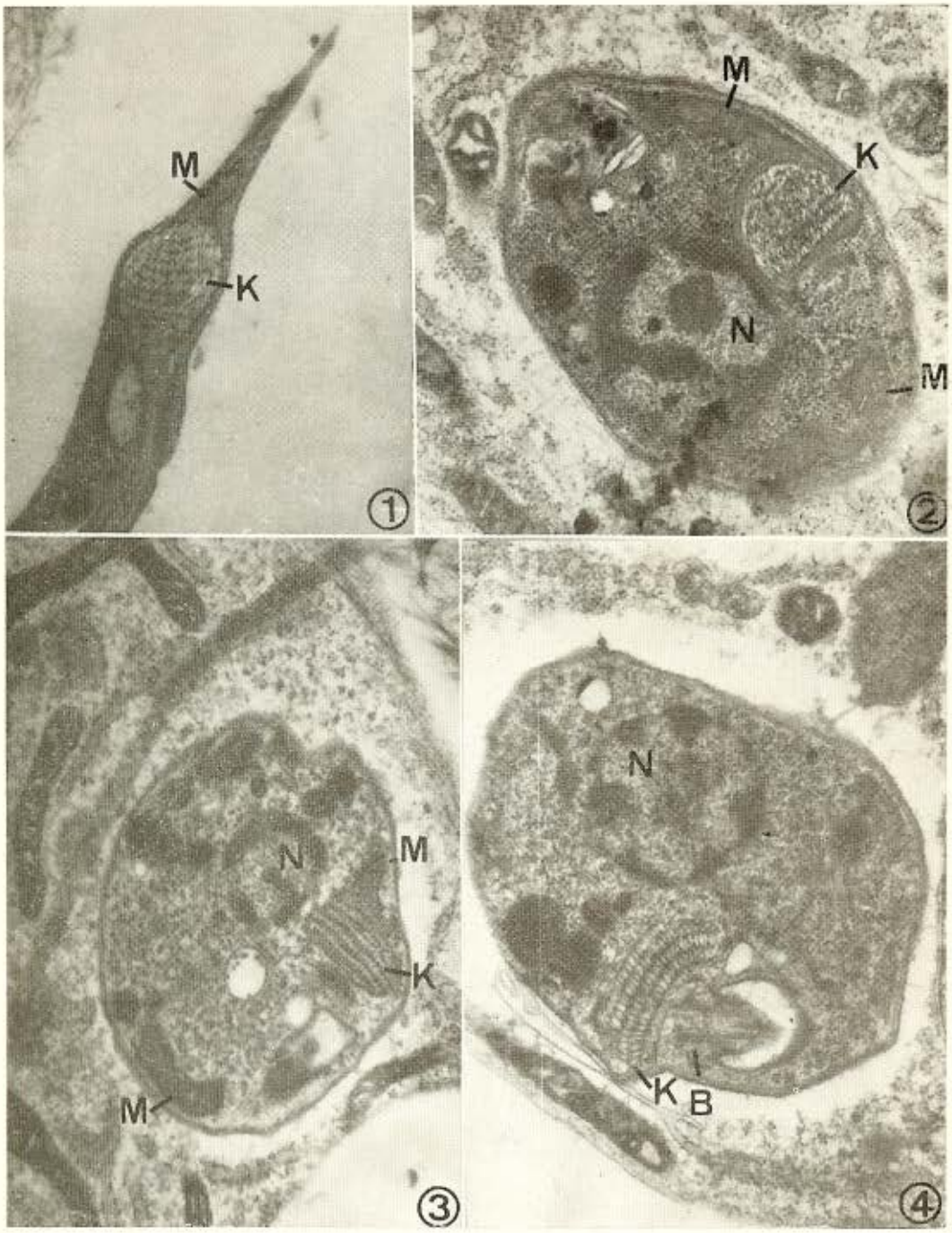

Fig. 1 — Free, extracellular trypanosome, showing basket-like structure in kinetoplast (K) which continues into mitochondrion (M). 21,500 ×. Fig. 2 — Part of trypanosome after recent penetration into new host cell. Kinetoplast (K) on way to anterior part of body at level of nucleus (N), seems to be located in enlargement of long mitochondrion (M). 28,500 ×. Fig. 3 — Part of trypanosome after recent penetration into new host cell. Kinetoplast (K) at level of nucleus (N) shows regular basket-like structure which continues into mitochondrion (M). 21,400 ×. Fig. 4 — Part of trypanosome after recent penetration into new host cell. Kinetoplast (K) with regular basket-like structure, already in anterior part of body. Basal body (B), nucleus (N). 28,500 ×.

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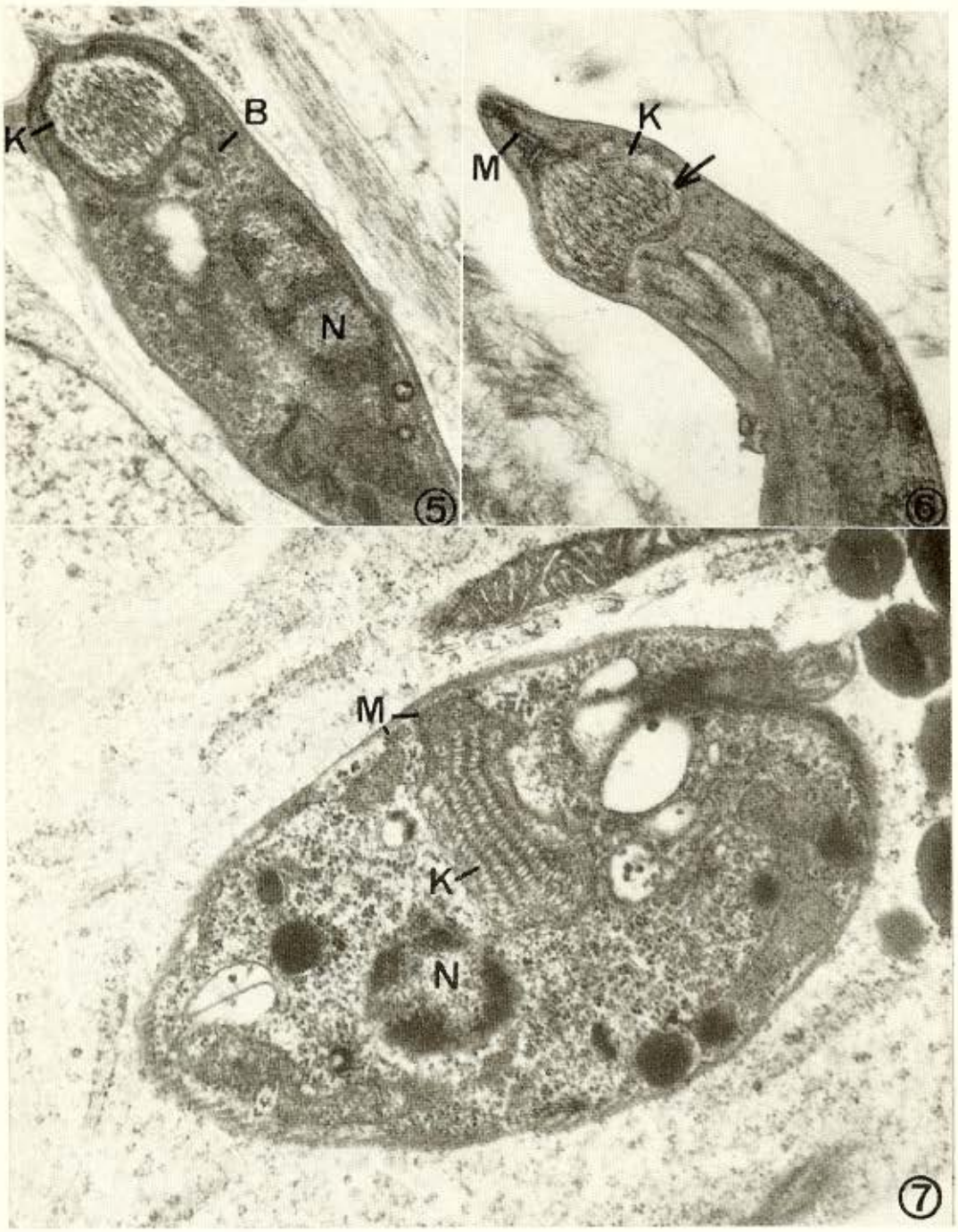

Fig. 5 — Part of trypanosome after recent penetration into new host cell, showing kinetoplast (K) with long irregularly shaped fibres. Nucleus (N), basal body (B). 28,500 ×. Fig. 6 — Part of free, extracellular trypanosome showing kinetoplast (K) with irregularly shaped fibres, in proximal part (↓) slight indication of horizontal structuration. Continues into mitochondrion (M) at pointed tip. 28,500 ×. Fig. 7 — Part of trypanosome after recent penetration into new host cell, showing kinetoplast (K) already in anterior part of body with rectangular shape and basket-like structure, small units of which are similar to mitochondrial cristae (M). Nucleus (N). 28,500 ×.

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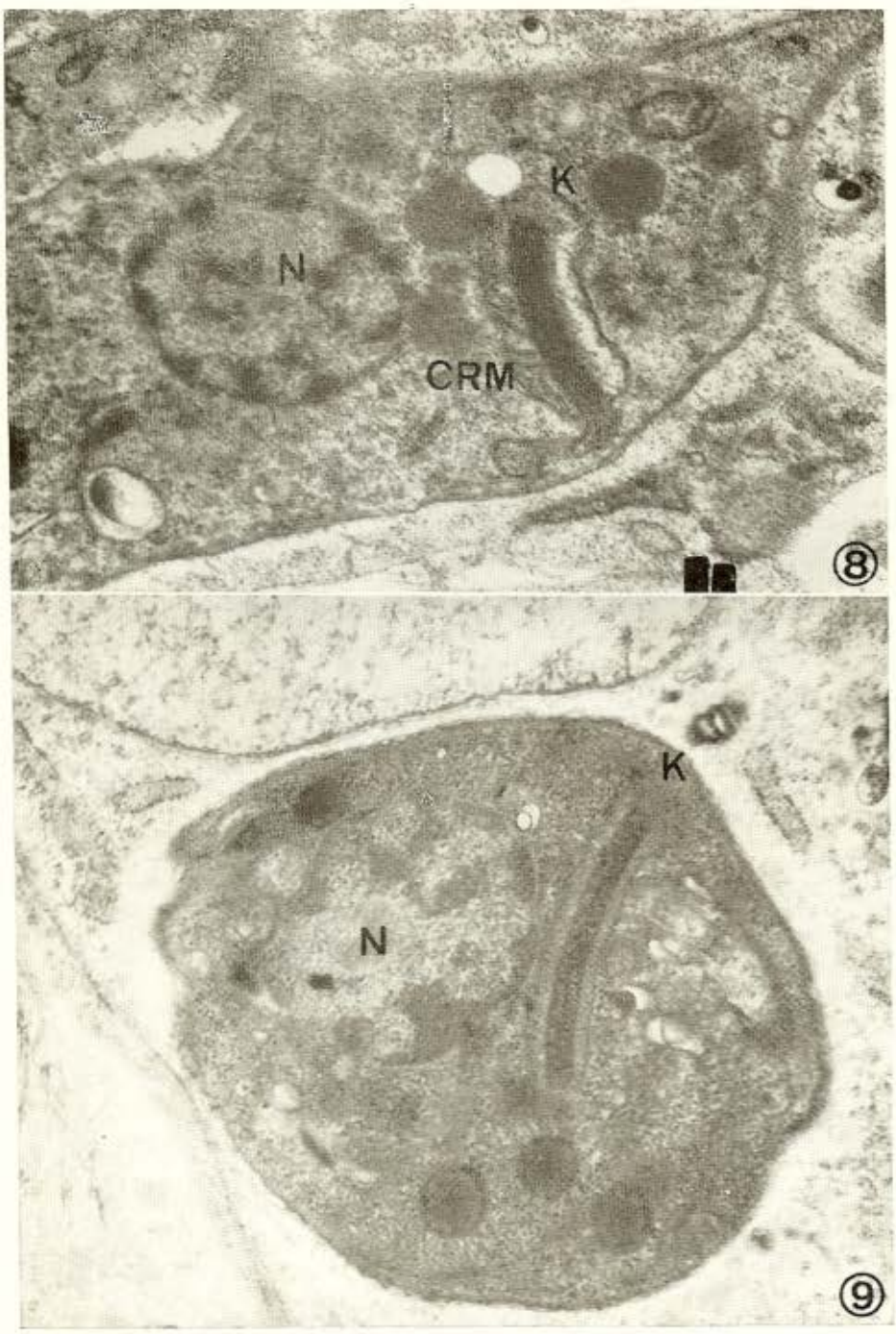

Fig. 8 — Leishmania body in cell which was filled with many parasites. Kinetoplast (X) showing osmiophilic mass and mitochondrial cristae (CRM) at periphery. 31,500 ×. Fig. 9 — Leishmania body in cell, showing horizontal line in kinetoplast (K), dividing osmiophilic mass in two rows. 28,500 ×.

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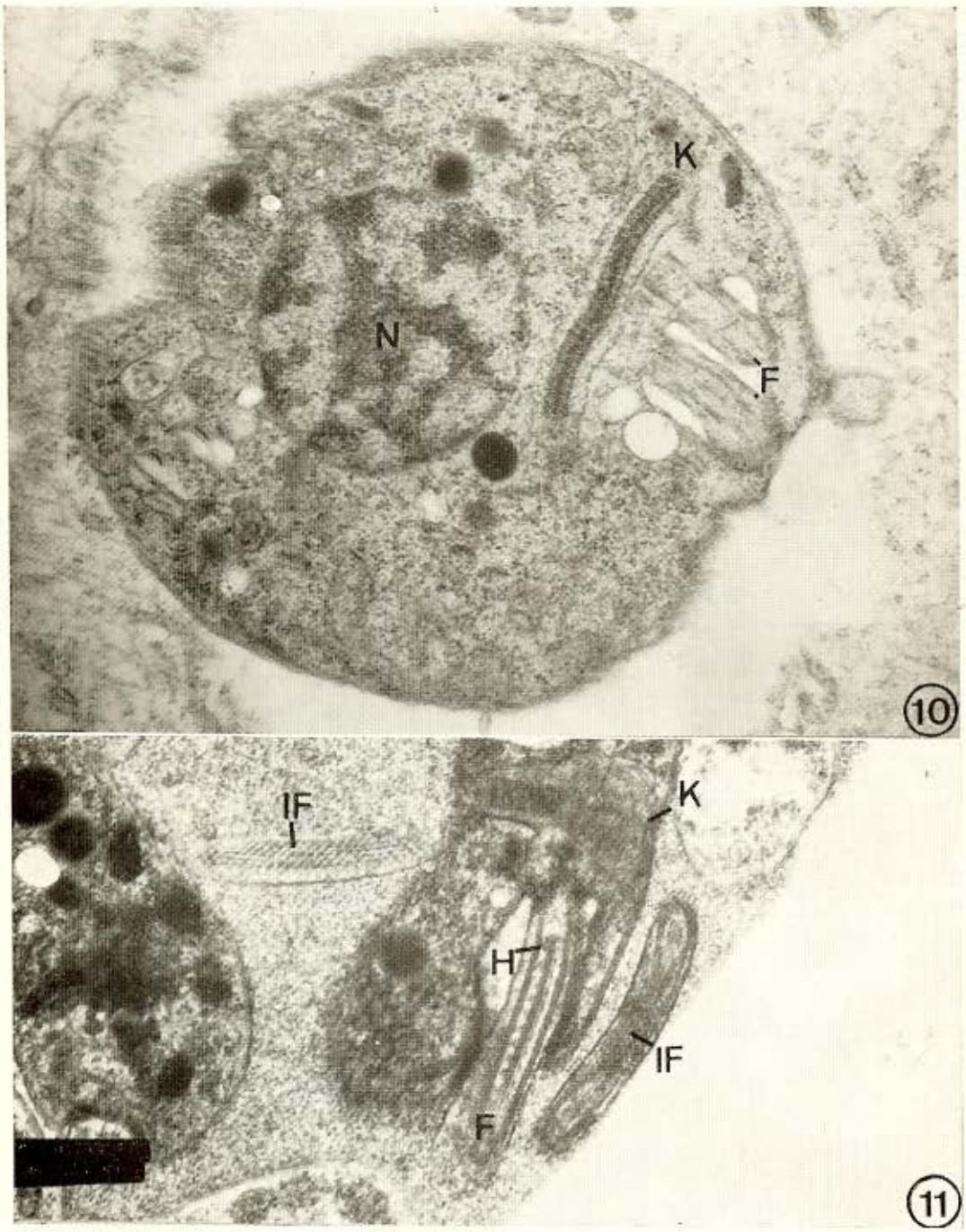

Fig. 10 — Leishmania body in cell at beginning of division, showing 2 flagellae (F). In kinetoplast (K) central, horizontal line dividing osmiophilic mass in two rows. 28,500 ×. Fig. 11 — Transition form of kinetoplast (K) in intracellular trypanosome at final phase of cycle, one side with typical osmiophilic, lamellar mass other side extending and forming long, irregularly shaped fibres. Flagellum (F) with helical substructure (H) around inner flagellar tubule. Lattice structure of intraflagellar body (IF) in segments of two flagellae. 28,500 ×.

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mitochondrial activity. What we are trying to find out is the way in which the mitochondria formation takes place.

The small units contained in the kinetoplast in orderly array, might represent the same cristae which are observed in the mitochondria with which the kinetoplast is continuous, and from which, in many images, they seem to pass into the tube-like mitochondrion itself. However, since the formation of mitochondrial cristae is usually performed by the inner mitochondrial membranes, it is possible that they do not represent cristae but have another significance, and the possibility that we are dealing here with a physiologically or genetically different form, cannot be ruled out as yet.

We do not know in which way the kinetoplast reproduces in the multiplying forms of the trypanosome. "*In vivo*" it can be observed easily that an invagination is formed in the middle of the thread-like structure in the leishmania bodies, which is followed by its division and by the binary division of the nucleus and the whole leishmania body. But we do not know, in which manner the osmiophilic mass, seen in the electron micrographs, increases.

It has been suggested by SANABRIA [11] and by MILDER & DEANE [6] for *Trypanosoma cruzi* and for *Trypanosoma conorhini* that this takes place by a horizontal division of the osmiophilic mass. This would explain the central line which has been observed by us in the kinetoplast of some of the leishmania forms. However, it could also be possible that this image represents the last phase of the reduction of the kinetoplast with those regular structures described above.

Summarizing the results which have been obtained so far, we can only state with certainty that two morphologically different forms exist among the free trypanosomes in the tissue cultures, both able to begin a new cycle in a new host cell. We are inclined to think that they represent the two last steps of the life cycle of the parasite, in which the one with the basket-like structure would be the final form into which the parasite may develop, but must not necessarily do so, in order to begin a new cycle.

If the small units which form the basket-like structure represent mitochondrial cristae, the kinetoplast proper in these forms would have transformed completely into a large mitochondrion and the opinion expressed by TRAGER [13], that the kinetoplast is a "highly specialized mitochondrion" would be confirmed. However, to clarify the real nature of this structure, more observations would have to be carried out.

RESUMO

Estudos sôbre a estrutura do cinetoplasto-condrioma do Trypanosoma (Schizotrypanum) cruzi *em cortes finos de culturas de tecido infetado*

Foi examinado com o microscópio eletrônico o cinetoplasto-condrioma do *Trypanosoma cruzi* em culturas de tecido, para estudar as transformações que ocorrem nesta estrutura durante as várias fases do seu ciclo evolutivo. O objetivo dêste estudo foi esclarecer o significado de uma estrutura nova, que foi observada recentemente no cinetoplasto de algumas formas adultas, extracelulares, em que as fibras de forma irregular, sempre descritas nestas formas, eram substituídas por pequenas unidades, parecidas com cristas mitocondriais, que ocuparam em arranjo muito regular tôda a região esférica do cinetoplasto.

Viu-se que ambas as estruturas observadas, a de fibras irregulares, sempre descrita, e a muito regular descrita recentemente, são de tripanosomas capazes de entrar nas células dos tecidos e iniciar um nôvo ciclo.

No início de um ciclo nôvo, quando o esférico cinetoplasto se transforma em uma estrutura mais lentiforme ou retangular, e migra para a porção anterior do parasita, a estrutura regular ainda pode ser reconhecida, não sendo, porém, mais encontrada quando muitas formas de leishmânia são contidas numa célula. O cinetoplasto mostra então a massa osmiófila, lamelar, que geralmente é descrita para esta forma. Em algumas formas de leishmânia foi observada uma linha horizontal na massa lamelar do cinetoplasto, que parece dividí-lo em duas filas horizontais, e que é considerada o último passo da redução de um cinetoplasto

que no tripanosoma adulto mostrou a estrutura regular.

Também nas formas adultas, no fim do ciclo intracelular, quando os tripanosomas estão ainda prêsos na célula hospedeira, a estrutura regular não foi encontrada. Supõe-se, por isso, que representa a estrutura do cinetoplasto no ponto culminante do ciclo evolutivo, para o qual o parasita pode evoluir, mas que não é necessário para iniciar um ciclo nôvo.

A natureza desta estrutura ainda está incerta; o seu possível significado no ciclo evolutivo do parasita está sendo discutido.

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