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# ACETYLATED ALPHA-TUBULIN IN *TRYPANOSOMA CRUZI:* IMMUNOCYTOCHEMICAL LOCALIZATION

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*We have used monoclonal antibodies specific for acetylated and non-acetylated* α*-tubulin to localize microtubules containing acetylated* α*-tubulin in all developmental forms of the life cycle of* Trypanosoma cruzi. *This was demonstrated using immunofluorescence and by transmission electron microscopy of thin sections, negative stained cells, and replicas of whole Triton X-100 extracted cells immunolabeled with antibody-gold complex. The antibody specific for acetylated* α*-tubulin (6-11B-1) binds to the flagellar, as well as to the sub-pellicular microtubules. The extent of labeling of the sub-pellicular microtubules with the monoclonal antibody recognized* α*-acetylated tubulin was smaller than that observed with the antibody which recognizes all tubulin isoforms. In relation to the developmental forms, the extent of labeling of the microtubules with antibody 6-11B-1 was larger in epimastigote and trypomastigote than in amastigote forms. Incubation of the parasites for 1 h at 0 °C or in the presence of either colchicine or vinblastine did not interfere with the sub-pellicular microtubules. These observations, in agreement with those reported for* Trypanosoma brucei brucei *(Schneider et al., 1987; Schulze et al., 1987; Sasse & Gull, 1988) indicate that the sub-pellicular microtubules of trypanosomatids represent stable microtubules containing acetylated a-tubulin (or the* $\alpha_3$*-tubulin isotype).*

Key words: *Trypanosoma cruzi* – acetylated tubulin – microtubules – cytoskeleton – immunocytochemistry

Protozoa of the Trypanosomatidae family, which includes agents of important diseases such as sleeping sickness, Chagas' disease and leishmaniasis, have two important groups of microtubules: those which form the flagellum and the sub-pellicular microtubules, which run along the parasite, and are located immediatelly below the plasma membrane (review in De Souza, 1984). It has been suggested that the sub-pellicular microtubules play an important role in the maintainance of the parasite's shape and that changes in their spatial distribution are involved in the process of differentiation which occurs during the life cycle of the trypanosomatids (Meyer & De Souza, 1976; De Souza, 1984). In view of these observations, the sub-pellicular microtubules, which can be considered as a subset of cytoplasmic microtubules, may represent an important target for drugs which, by interacting with and disrupting this structure, could interfere with the parasite's viability.

The primary components of microtubules are the heterodimers of alpha- and beta-tubulin. There are multi-gene families of both α- and β-tubulin (Cleveland, 1983; Cleveland & Sullivan, 1985). There is good evidence to suggest that expression of these tubulin multigene families can lead to the presence of multiple α- or β-tubulin polypeptides within individual cells. Tubulin may also be altered functionally as a result of post-translational modifications. These include: (a) phosphorylation of β-tubulin (Eipper, 1972); (b) detyrosinolation (Argarana et al., 1980; Matersen, 1982; Sherwin et al., 1987) and re-tyrosinolation (Barra et al., 1973; Arce et al., 1975; Raybin & Flavin, 1975, 1977; Thompson, 1982) of α-tubulin and (c) acetylation of α-tubulin (L'Hernault & Rosembaum, 1983, 1985a, b). The substrate for the enzymes that perform such modifications is α-tubulin in the polymerized state (Thompson et al., 1979).

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Acetylation of α-tubulin, first detected in *Chlamydomonas* (L'Hernault & Rosenbaum, 1983), is one form of post-translational modification that seems to be involved in determining microtubule stability. The identification and localization of acetylated α-tubulin became possible with the production by Piperno & Fuller (1985) of a monoclonal antibody, designated as 6-11B-1, that is specific for this form of tubulin. Subsequent studies carried out in *Chlamydomonas* (Le Dizet & Piperno, 1986) and *Physarum polycephalum* (Diggins & Dove, 1987; Sasse et al., 1987; Sasse & Gull, 1988) have shown that besides the axonemal microtubules, some cytoplasmic microtubules also contain acetylated tubulin. A subset of cytoplasmic microtubules containing acetylated α-tubulin was also seen in some vertebrate cells in culture (Piperno et al., 1987).

We have used two monoclonal antibodies to detect acetylated and unacetylated forms of α-tubulin in epimastigote, amastigote and trypomastigote forms of the pathogenic protozoan *Trypanosoma cruzi.* The two α-tubulin forms were located using immunofluorescence and electron microscopy of thin sections, negatively stained cells, as well as replicas of Triton X-100 extracted cells immunolabeled with antibody-gold complex. The tubulins were also detected in immunoblottings. Our observations show that the sub-pellicular microtubules of trypanosomatids contain acetylated α-tubulin and that the extent of acetylation, as analyzed by quantification of gold particles seen in whole negatively stained cytoskeletons, varies according to the developmental stage of the parasite's life cycle. These data, together with those showing no morphological alterations in the sub-pellicular microtubules of *T. cruzi* incubated at 4 °C or in the presence of colchicine or vinblastine indicate that the sub-pellicular microtubules are stable structures.

## MATERIALS AND METHODS

*Parasites* – Epimastigote forms of *T. cruzi* (Y strain) were cultivated for four days at 28 °C in a liver infusion-tryptose medium (Camargo, 1964). Trypomastigote forms were obtained from the supernatant of cell cultures previously infected with bloodstream forms, as described elsewhere (Carvalho & De Souza, 1983). The parasites were collected by centrifugation at 1000 g for 10 min and washed three times in 0.01 M phosphate-buffered saline (PBS), pH 7.2, before the experiments.

*Parasite treatments* – Stock solutions (1 mg/ml) of colchicine and vinblastine (Sigma Chemical Company) were prepared in PBS, pH 7.2. The cells were incubated for 10, 30 or 60 min at room temperature in the presence of 10 μg/ml colchicine or vinblastine and immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. In other experiments, cells were incubated for 60 min at 0-2 °C and immediatelly fixed with a cold glutaraldehyde solution. In a third type of experiment, parasites were disrupted as described previously (Dwyer, 1980), centrifuged at 750 g for 10 min and the pellet was fixed in glutaraldehyde solution. Fixed cells were then processed for routine transmission electron microscopy.

*Antibodies* – The mouse monoclonal antibodies 6-11B-1 and B-5-1-2 were kindly supplied in culture medium by Dr G. Piperno (The Rockfeller University, New York, USA). The antibody 6-11B-1 is specific for acetylated α-tubulin (Piperno & Fuller, 1985) and antibody B-5-1-2 recognizes all α-tubulin isoforms from *Chlamydomonas,* sea urchin, and human tubulin (Piperno et al., 1987). A polyclonal anti-tubulin rabbit antibody (Milles Yeda, Rehovot, Israel) was used in some experiments.

*Immunofluorescence* – Parasites were fixed for 30 min at room temperature in 3.7% formaldehyde (freshly prepared from paraformaldehyde), then washed three times with PBS and allowed to adhere for 15 min to coverslips previously coated for 15 min with 0.1% poly-L-lysine (MW-40,000, Sigma Chemical Company, USA). The adherent cells were incubated for 30 min in 50 mM $NH_4Cl$ to block free aldehyde groups, washed three times with PBS and then treated with 0.1% Triton X-100 (in PBS) for 10 min at room temperature. A 30 minute-incubation with PBS-1% bovine serum albumine (PBS-A) was used to prevent non-specific antibody binding. Incubation of the cells with antibodies in PBS-A was performed for 60 min at room temperature. Subsequently, the coverslips were washed several times in PBS-A and incubated for 60 min with either fluorescein-labeled goat anti-mouse (for the monoclonal antibodies) or fluorescein-labeled goat anti-rabbit (for the polyclonal antibody) antibodies (both from Sigma Chemical Company, USA; diluted 1:50). Control cells were incubated only with the fluorescein-labeled antibodies. After incubation, the coverslips were washed three times in PBS, inverted onto a slide over a drop of 90% glycerol, 10%

PBS, 0.1% n-propyl-gallate (pH 7.2), and maintained in place with nail varnish. Photomicrographs were obtained using a Zeiss Universal Photomicroscope equipped with an epi-fluorescence condenser and a 63 X Planapo objective. The exposure times varied from 30 to 60 s. A Kodak Tri-x Pan film was used and developed in a Kodak HC-110 D developer.

*Electron microscopy* – For thin sections, washed parasites were treated for 1 min at room temperature with 1.0% Triton X-100 before fixation for 30 min in a 0.1% glutaraldehyde – 4% formaldehyde solution in 0.1 M cacodylate buffer, pH 7.2 containing 0.1% Triton X-100. After fixation, the cytoskeletons were incubated for 60 min in a 50 mM $NH_4Cl$ solution in PBS, to block free aldehyde groups, washed three times in PBS-A and then incubated for 1 h at room temperature in the presence of anti-tubulin antibodies (diluted 1:10). Thereafter the cytoskeletons were washed five times in PBS-A and incubated for 1h at room temperature with a goat anti-mouse IgG-20 nm gold conjugate solution (Janssen, Belgium; diluted 1:20). Thereafter, they were washed with PBS, post-fixed for 1 hr in 1% $OsO_4$, dehydrated in ethanol and embedded in Epon.

For observation of the whole cytoskeleton, living parasites were allowed to adhere to 200 mesh formvar-carbon-coated and poly-L-lysine-treated gold grids as described for immunofluorescence. The adherent cells were then exposed for 10 min at room temperature to 1.0% Triton X-100 and then fixed in a 0.1% glutaraldehyde – 4% paraformaldehyde solution (De Souza & Benchimol, 1984). After fixation, the grids were successively washed with PBS-A, incubated in $NH_4Cl$, incubated for 60 min in the presence of the anti-tubulin antibodies, washed five times with PBS-A, incubated in the presence of the gold conjugate, washed several times with PBS and negatively stained with 1% phosphotungstic acid. In some experiments, a suspension of parasites containing amastigote, epimastigote, and trypomastigote forms was allowed to adhere to the grids and immunolabeled as described above. The number of gold particles per square micrometer of the whole cytoskeleton was determined in at least ten cells.

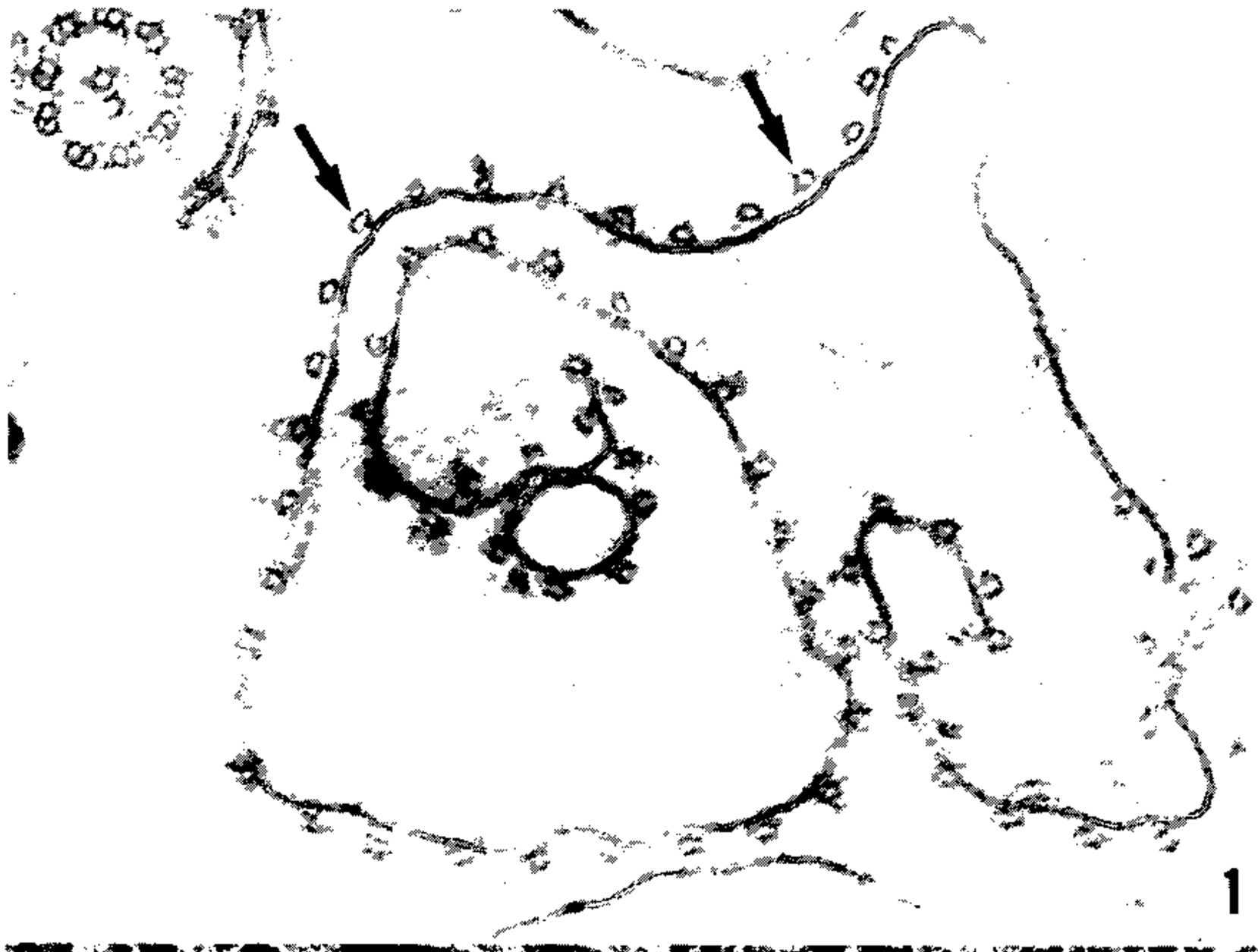
For observation of replicas of the cytoskeleton, the parasites were allowed to adhere to glass coverslips and processed as described for the negative staining preparations. After incubation with the gold conjugate, the coverslips were washed, dehydrated in ethanol and critical point dried according to standard techniques. Unidirectional platinum-carbon shadowing at 45°, and carbon replication at 90° were carried out in a Balzers freeze-etch unit at a vacuum of $1.10^{-6}$ torr. Replicas were released in 20% hydrofluoric acid, cleaned in 20% $H_2SO_4$ and distilled water and collected on 200 mesh copper grids.

Replicas of quick-frozen, freeze-fractured and deep-etched cells were prepared as described previously (Souto-Padrón et al., 1984).

Thin sections, negatively stained preparations and replicas were examined in a Jeol 100 CX electron microscope operated at 60 to 100 KV.

*Polyacrilamide gel electrophoresis and immunoblotting* – Epimastigotes were washed three times in PBS containing 2 mM phenylmethylsulphonyl fluoride and 100 µg/ml leupeptin (PBS-PMSF-leup), resuspended in the same buffer to 2 mg/ml of total protein, evaluated according to Lowry et al. (1951), and lysed with an equal volume of double concentrated Laemmli's (1970) sample buffer. Cytoskeletons were prepared by treating washed epimastigotes with 1% Triton X-100 in PBS-PMSF-leup for 10 min at room temperature before lysis in the same conditions as epimastigote whole cells. Samples were boiled for five minutes and resolved in 5% to 15% acrylamide gradients according to the method of Laemmli (1970). Molecular weights werre calibrated using high molecular weight markers from Bio-Rad Laboratories (Richmond, California) and purified tubulin from rabbit brain. Gels were stained for protein with 0.1% Coomassie Brilliant Blue-R in 50% trichloroacetic acid (TCA) and destained in 10% acetic acid.

Transfer of protein bands from gels to nitrocellulose sheets was accomplished as described (Towbin et al., 1979). PBS pH 8.0 containing 5% non-fat milk (Johnson et al., 1984) was used to block the nitrocellulose sheets and to dilute the antibodies. The blots were incubated with antibodies against acetylated (diluted 1:200) or non-acetylated (diluted 1:500) α-tubulin for 2 hr at 37 °C. After incubation with secondary antibodies conjugated with peroxidase (Byosis, France, diluted 1:100) for 2 hr at 37 °C, reacting bands were visualized by the diaminobenzidine method (Tsang et al., 1983).



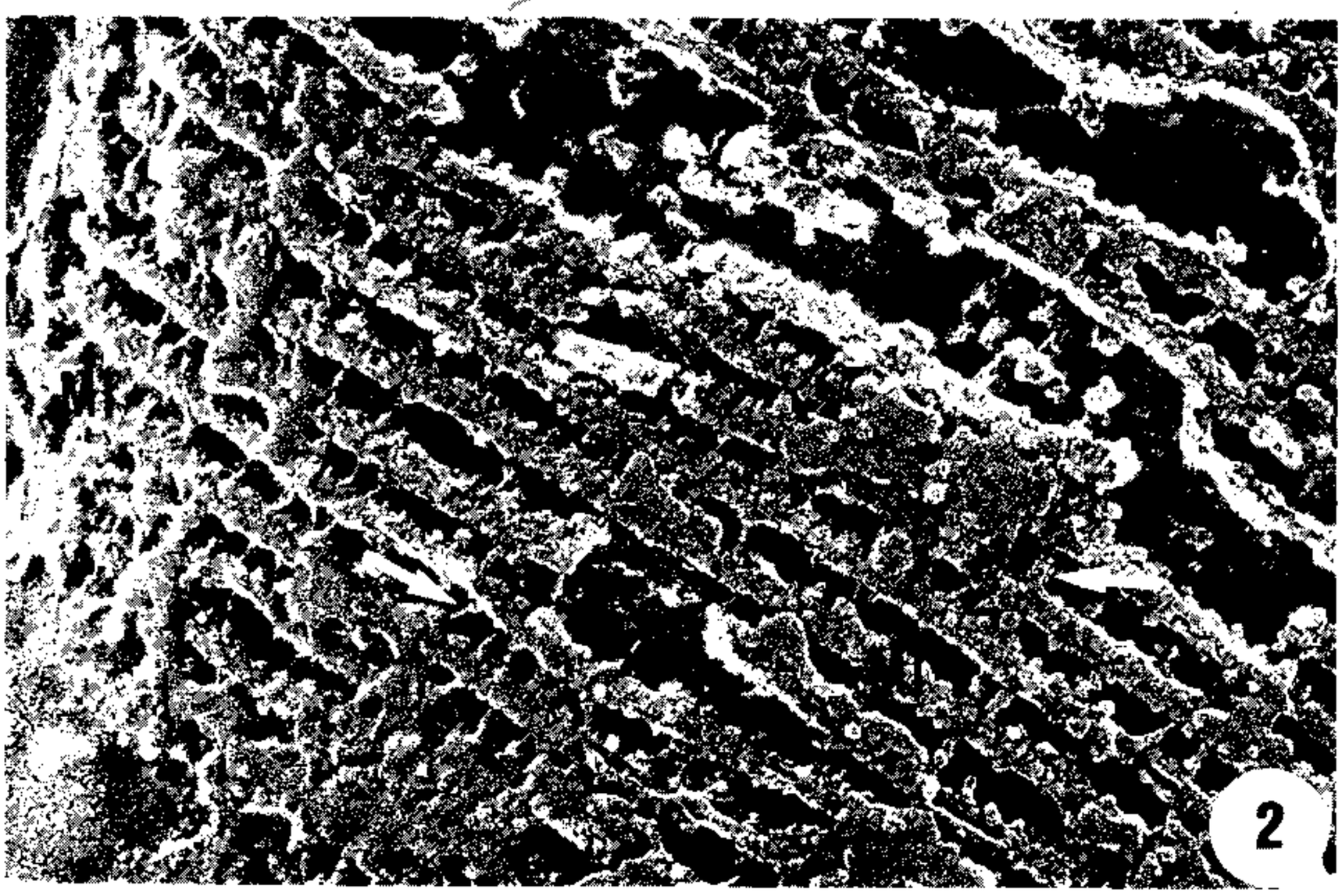




Fig. 1: thin section of a pellet obtained after disruption of the cells and centrifugation at 750 g for 10 min. The sub-pellicular microtubules (arrows) remain associated with the plasma membrane. X 52,000. Fig. 2: parasite treated with Triton X-100 immediately before glutaraldehyde fixation and replicated using rotary shadowing at low angle. Most of the plasma membrane was removed, revealing the underlying layer of sub-pellicular microtubules (MT). Fibrils can be seen connecting the sub-pellicular microtubules to each other (arrows). X 100,000.

## RESULTS

*Effect of temperature, colchicine and vinblastine* – Incubation of epimastigotes, amastigotes and trypomastigotes of *T. cruzi* at 0 °C for 60 min did not alter the morphology of the parasites, as seen by phase contrast microscopy. Similar observations were made in parasites which had been incubated for 60 min at 0 °C in the presence of colchicine or vinblastine (not shown).

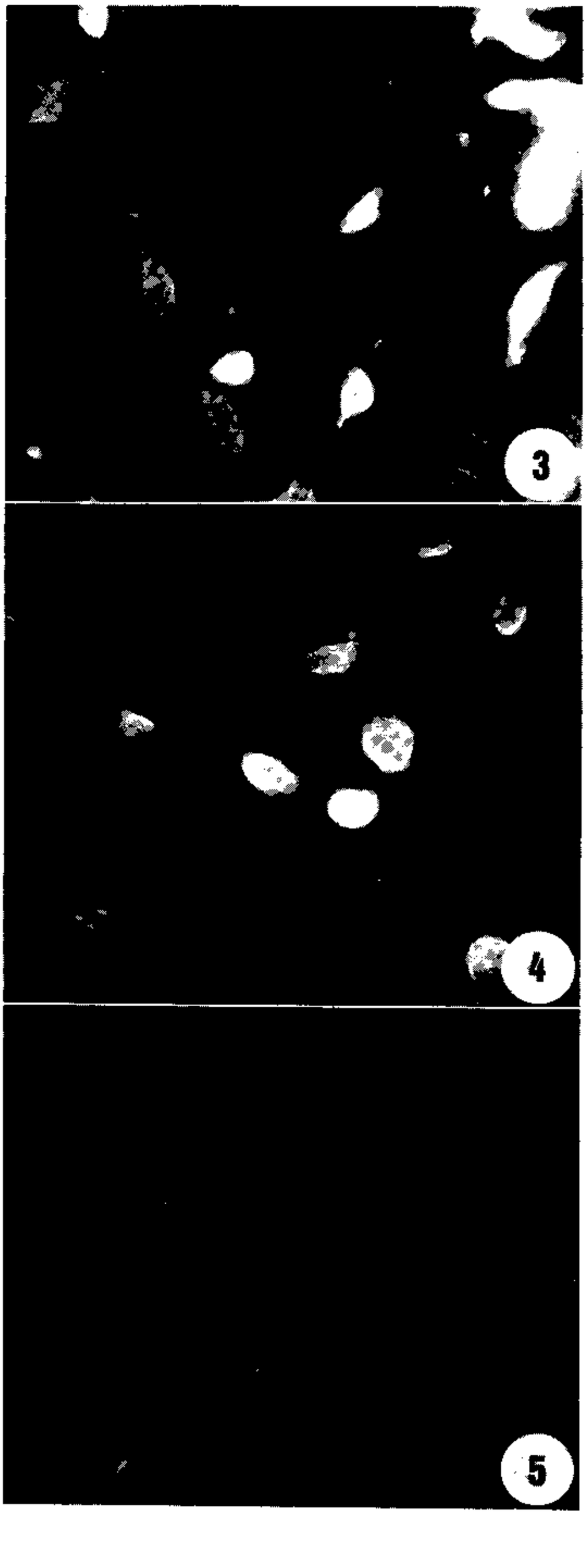
Transmission electron microscopy of thin sections of control, cold-, colchicine-, or vinblastine-treated parasites showed a similar distribution of the sub-pellicular microtubules. They showed a parallel array, being separated from each other by a distance of 44 nm (center

to center) and separated from the plasma membrane by a distance of 15 nm. Even in disrupted cells the sub-pellicular microtubules remained associated with the plasma membrane (Fig. 1). As shown previously (Souto-Padrón et al., 1984) in replicas of quick frozen, freeze-fractured and deep-etched cells, filamentous-bridges connected the microtubules to each other (Fig. 2).

*Immunofluorescence* – Three anti-tubulin antibodies were used to analyse the localization of microtubules in *T. cruzi*, one polyclonal antibody and two monoclonal antibodies: B-5-1-2 which recognizes all α-tubulin isoforms and 6-11B-1 which is specific for acetylated α-tubulin (Piperno & Fuller, 1985). Our observations show that the pattern of immunofluorescence of the parasites was the same for the three antibodies used (Figs 3-5). However, it was more intense when the polyclonal antibody was used (Fig. 3). Fluorescence was localized in the flagellum as well as in the cell body. In view of the well known presence of acetylated α-tubulin in the flagella of other cell types we focused our observations on the cell body.

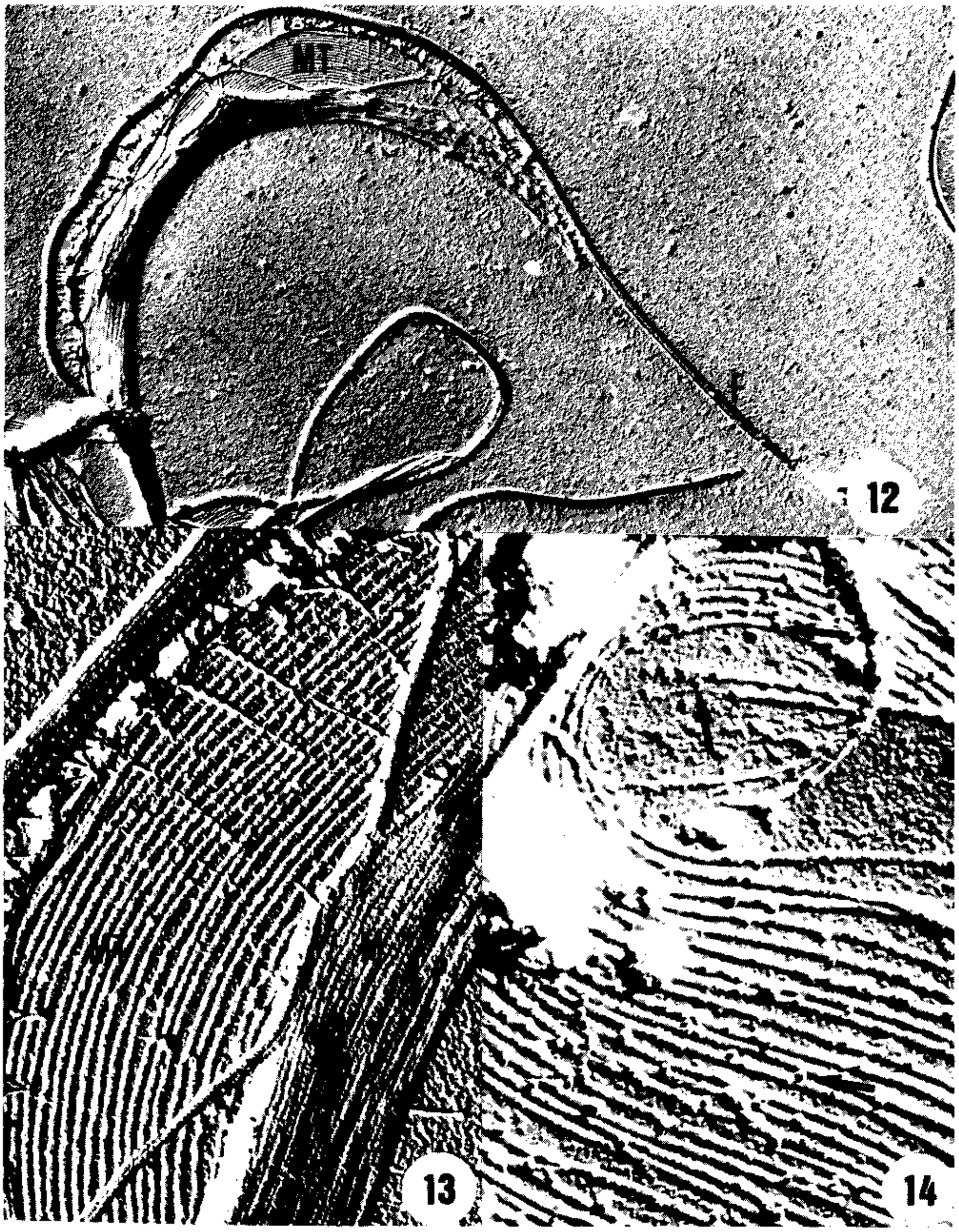
*Electron microscopy* – Examination of thin sections of Triton X-100 treated cells incubated initially in the presence of anti-tubulin antibodies and subsequently with gold conjugated antibody, showed the presence of gold particles associated with the sub-pellicular (Fig. 7) and flagellar (Fig. 8) microtubules. Probably due to problems related to the access of the gold-labeled antibodies even in Triton-X extracted cells, some microtubules were not labeled.

Examination of the cytoskeleton of the whole negatively stained parasite showed intense labeling of all portions of the cell body and flagellum of *T. cruzi* (Figs 6, 9-11). In these preparations the anterior and posterior regions of the cell body, as well as the regions where the nucleus and the kinetoplast were located, could be easily identified. The analyses of a large number of cells incubated with monoclonal antibodies which recognize either all α-tubulin isoforms or specifically, only acetylated α-tubulin, did not show any difference in the extent of the labeling of the flagellar microtubules. No labeling was observed when the preparations were incubated only in the presence of the gold-labeled antibody. In relation to the sub-pellicular microtubules, a quantitative analysis of the number of gold




Figs 3-5: immunofluorescence microscopy of parasites incubated in the presence of fluorescein labeled second antibody after previous incubations of Triton X-100 treated cells in the presence of a polyclonal anti-tubulin antibody (Fig. 3) or monoclonal antibodies which recognize either all isoforms of α-tubulin (Fig. 4) or only acetylated α-tubulin (Fig. 5). In all cases the cell body and the flagellum are labeled. X 900.


Figs 6, 8-9 and 10-11: negative stain of whole parasites adhered to formvar and poly-L-lisine-coated grids, extracted with Triton X-100 incubated in the presence of the monoclonal antibody which recognizes acetylated α-tubulin and then with a goat anti-mouse gold complex. Gold particles can be seen in association with the sub-pellicular microtubules (Figs 6, 10, 11). The linear disposition of the gold particles is evident. Fig. 6: X 17.500; Fig. 10: X 34,000; Fig. 11: X 30,000. Fig. 7: thin sections of parasites exposed for 1 min to 1% Triton X-100 before glutaraldehyde fixation and subsequently incubated in the presence of the monoclonal antibody which recognizes acetylated α-tubulin and then with goat anti-mouse gold complex. Gold particles can be seen in association with the sub-pellicular microtubules (arrows). X 35,000. Fig. 8: thin sections of parasites exposed for 1 min to 1% Triton X-100 before glutaraldehyde fixation and subsequently incubated in the presence of the monoclonal antibody which recognizes non-acetylated α-tubulin and then with a goat anti-mouse gold complex. Gold particles are seen in association with peripheral microtubules of the axoneme. X 56,000. Fig. 9: negative staining of whole parasites adhered to formvar and poly-L-lysine coated grids, extracted with Triton X-100, incubated in the presence of the monoclonal antibody which recognizes non acetylated α-tubulin and then with a goat anti-mouse gold complex. Gold particles can be seen in association with the axonemal microtubules. X 45,000.




Figs 12-13: replicas of whole parasites adhered to poly-L-lysine coated glass coverslips, then treated with Triton X-100, fixed with glutaraldehyde, dehydrated and critical point dried. Part of the plasma membrane (M) was removed exposing the sub-pellicular microtubules (MT). F, flagellum. Fig. 12: X 7,300; Fig. 13: X 20,000. Fig. 14: replica of a whole parasite treated as described for Figs 12 and 13. However, the cytoskeleton was incubated first in the presence of the monoclonal antibody which recognizes acetylated $\alpha$-tubulin and subsequently with goat anti-mouse gold complex. Gold particles are seen associated to the sub-pellicular microtubules (arrows). X 25,000.

particles per square micrometer of the whole cytoskeleton of cells simultaneously processed showed that the extent of labeling varied according to the antibody used and according to the developmental stage of the parasite's life cycle (Table). For all forms, labeling was more intense when the monoclonal antibody B-5-1-2 was used. The extent of acetylation of sub-pellicular microtubules, as assayed by the immunocytochemical method, was larger in epimastigote and trypomastigote than in amastigote forms.

TABLE

Number of gold particles per square micrometer of the whole cytoskeleton of immunolabeled and negatively stained parasites[a,b]

| Developmental Stages | Density of gold particles on cells Immunolabeled with antibodies B-1-1-2 | 6-11B-1 |
|---|---|---|
| Amastigotes | 101 ± 38 | 18 ± 5.1 |
| Epimastigotes | 124 ± 18 | 52 ± 11 |
| Trypomastigotes | 182 ± 24 | 46 ± 25 |

*a*: data obtained in experiments in which all developmental stages were adhered to the same grid.
*b*: at least ten cells of each developmental form were counted on each grid.

The parallel array of the sub-pellicular microtubuls of *T. cruzi* could be well visualized in replicas of cells which first were attached to a glass coverslip, then treated with Triton X-100, fixed with glutaraldehyde, dehydrated and critical point dried (Figs 12-13). Using this technique the general form of the parasite was maintained (Fig. 12). When, this technique was used for cells incubated in the presence of the anti-tubulin antibodies and subsequently with a gold-conjugated second antibody, the gold particles remained associated with the replicas and were seen in association with the sub-pellicular microtubules (Fig. 14).

*Polyacrylamide gel electrophoresis and immunoblotting* – Comparing electrophoretic profiles of epimastigote whole cells and epimastigote cytoskeletons containing the same amount of protein (50 µg) we have observed that tubulin is the major protein of both samples (Fig. 15). Nevertheless, in the cytoskeletons tubulin was even more enriched in relation to the total proteins of this sample. Other major bands of the cytoskeletons profile, corresponding to proteins of 81, 78, 75, 49, 47, 31 and 28 kDa, besides several minor bands, could be observed. A protein band of 43 kDa, which should correspond to actin, was remarkable on the whole cell profile, but very faint on the cytoskeletons. Previous studies (Mortara, 1989) have demonstrated that actin remained associated with the cytoskeleton when it was prepared with Nonidet P-40 in PIPES (piperazine-N,N'-bis [2-ethane sulfonic acid]) although it was not possible to observe actin filaments.

Antibodies against non-acetylated α-tubulin recognized very strongly tubulin from whole epimastigotes cells (Fig. 16A) as well as tubulin from rabbit brain (Fig. 16B). The monoclonal antibody against acetylated α-tubulin also recognized tubulin from whole epimastigote cells (Fig. 16C), but it was necessary a higher antibody concentration for a good reaction. This antibody presented a very weak reaction with purified brain tubulin (Fig. 16D).

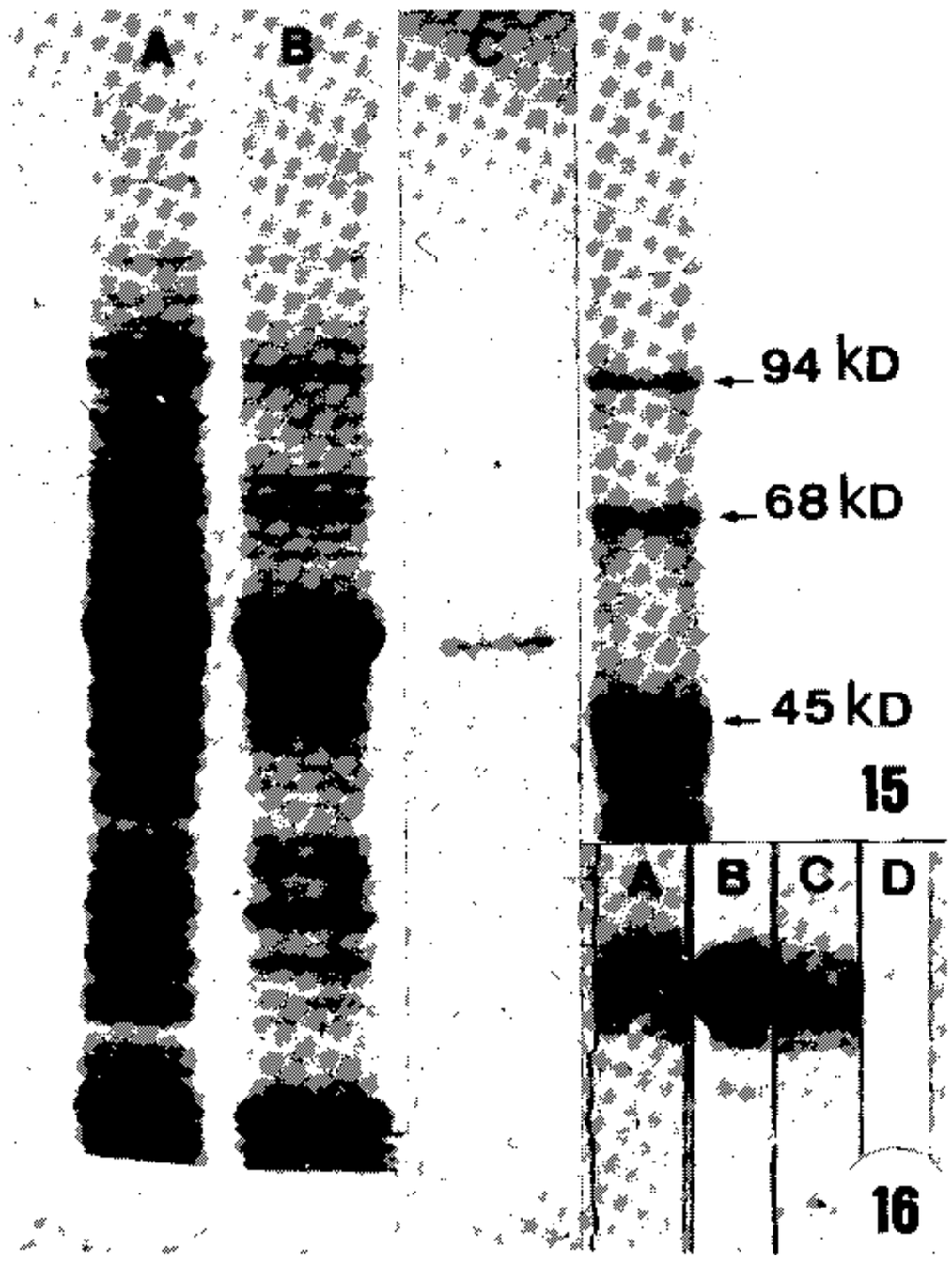



Fig. 15: SDS-PAGE analysis of whole epimastigotes of *Trypanosoma cruzi* (lane A), cytoskeleton preparation (lane B) and purified tubulin from rabbit brain (lane C). Molecular weight markers are indicated by arrows. Fig. 16: immunoblots showing the binding of the monoclonal antibodies B-5-1-2 (which recognizes all isoforms of α-tubulin) to whole epimastigotes (lane A) and purified tubulin (lane B) and 6-11B-1 (which recognizes acetylated α-tubulin) to whole epimastigotes (lane C) and purified tubulin from rabbit brain (lane D).

## DISCUSSION

The sub-pellicular microtubules of trypanosomatids represent one of the first systems of organized cytoplasmic microtubules described (Meyer & Porter, 1954). In view of their possible participation in determining the shape of the parasite, which varies significantly during its life cycle, it has been assumed that the sub-pellicular microtubules of trypanosomatids may constitute a target for the chemotherapy of the important diseases caused by protozoa of the Trypanosomatidae family (reviewed in De Souza, 1984).

Our observations as well as those reported by others (Angelopoulos, 1970; Messier, 1971; Hunt & Ellar, 1974; De Souza, 1976; Meyer & De Souza, 1976; Dwyer, 1980) indicate that the sub-pellicular microtubules represent a special subset of cytoplasmic microtubules: (a) incubation of epimastigotes of *T. cruzi* at 0 °C, a condition in which most of the cytoplasmic microtubules of eucaryotic cells are depolymerized, did not interfere with the sub-pellicular microtubules; (b) incubation of the parasites in the presence of colchicine or vinblastine, at concentrations which interfere with most of the cytoplasmic microtubules of vertebrate cells, did not interfere with the sub-pellicular microtubules. Similar observations were previously reported in *Crithidia fasciculata* (Messier, 1971). When used at concentrations twenty times higher than usually employed, however, these drugs interfere with the sub-pellicular microtubules (Ono & Nakabayashi, 1979); (c) in intact cells the sub-pellicular microtubules are not isolated but connected to each other and to the plasma membrane by short filaments whose composition has not yet been determined but which can be clearly visualized, mainly in replicas of rapidly frozen, freeze-fractured and deep-etched cells (Souto-Padrón et al., 1984). The membrane-microtubule linkage is so strong that these two structures remain associated when the trypanosomatids are ruptured and a plasma membrane fraction is isolated by differential centrifugation (Hunt & Ellar, 1974; De Souza, 1976; Dwyer, 1980). Taken together, the behavior of the sub-pellicular microtubules of trypanosomatids in conditions in which most of the cytoplasmic microtubules of other eucaryotic cells are disrupted indicate that they represent stable microtubules. It is important to point out, however, that the sub-pellicular microtubules of trypanosomatids are depolymerized when in presence of a high (100 μm) $Ca^{++}$ concentration (Dolan et al., 1986) or when incubated in the presence of phenothiazines (Seebeck & Gehr, 1983).

Previous biochemical studies have shown that all isotypes of α-tubulin are present in trypanosomatids (Bordier et al., 1982; Russell & Gull, 1984; Russell et al., 1984; Steiger et al., 1984; Schneider et al., 1987; Sherwin et al., 1987; Sasse & Gull, 1988). Analysis of the flagellar and sub-pellicular microtubules in *Crithidia fasciculata* showed that the $\alpha$-$_3$ isotype was the only isotype of $\alpha_3$-tubulin found in the flagellum and that it appeared in small amounts in the sub-pellicular microtubules (Russell et al., 1984). Our present results, however, show that the monoclonal antibody B-5-1-2, which recognizes all α-tubulin isoforms, binds to flagellar microtubules of *T. cruzi* similarly to the monoclonal 6-11-B-1, which is specific for the acetylated form of α-tubulin. Evidence was obtained showing that, as reported for other cells types (L'Hernault & Rosenbaum, 1983, 1985a, b; Russell et al., 1984), $\alpha_3$-tubulin in trypanosomatids is a post-translation modification of $\alpha_1$, the major α-tubulin of the tubulin cytoplasmic pool. Some evidence has been obtained in the last years showing that the $\alpha_3$ isotype of α-tubulin corresponds to the acetylated form of α-tubulin. These evidences include (a) the observation that only the $\alpha_3$ isotype appears labeled when cells are radiolabeled with $^3$H-acetate under conditions of stringent protein synthesis inhibition and subsequent analysis of the proteins by two-dimensional gel electrophoresis (Schneider et al., 1987); (b) a monoclonal antibody which specifically recognizes acetylated α-tubulin when used in immunoblots recognized only the $\alpha_3$ tubulin isotype, as seen in two dimensional gel electrophoresis (Diggins & Dove, 1987; Sasse et al., 1987). Acetylation occurs in the ε-amino group of a lysine residue in the α-tubulin polypeptide (L'Hernault & Rosenbaum, 1985a, b) in an enzymatic process involving the participation of an α-tubulin acetylase found in the flagella of *Chlamydomonas* (Greer et al., 1985; Maruta et al., 1985).

The production of a monoclonal antibody which recognizes specifically acetylated α-tubulin made it possible to analyse the distribution of microtubules which contain acetylated tubulin, e.g., the $\alpha_3$ tubulin isotype. Our immunocytochemical observations using the 6-11B-1 monoclonal antibody show clearly that the sub-pellicular microtubules of *T. cruzi* contain acetylated tubulin and are in agreement with the biochemical results reported by Schneider et al. (1987) showing that $^3$H-acetate is incorporated into the $\alpha_3$ tubulin isotype found both in the flagellum and in the sub-pellicular microtubules of *T. brucei.*

We observed, using gold-antibody complex and electron microscopy of the whole cytoskeleton of *T. cruzi,* an intense labeling of the sub-pellicular microtubules of cells previously incubated with the monoclonal antibody 6-11B-1, which specifically recognizes acetylated α-tubulin. However, the extent of the labeling

was much smaller than that observed when the parasites were incubated in the presence of the monoclonal antibody B-5-1-2, which recognizes all α-tubulin isoforms. It is interesting to notice that the extent of labeling of the sub-pellicular microtubules with the antibody specific to acetylated α-tubulin was significantly smaller in amastigote than in epimastigote and trypomastigote forms.

Taking together (a) the results obtained by us and by previous authors (Dolan et al., 1986; Messier, 1971; Ono & Nakabayashi, 1979) on the response of the sub-pellicular microtubules to microtubule-disrupting drugs, (b) our immunocytochemical observations and (c) the biochemical observations recently reported by Schneider et al. (1987), we can conclude that the sub-pellicular microtubules of trypanosomatids correspond to stable cytoplasmic microtubules, containing acetylated (or $\alpha_3$) alpha tubulin. A direct correlation has been made in the literature (Piperno & Fuller, 1985; Cambray-Deakin & Burgoyne, 1987; Schulze et al., 1987) between the presence of acetylated α-tubulin and microtubule stability. Acetylation has been detected in microtubules of the flagella of many cell types (Piperno & Fuller, 1985) and in *T. brucei* it was detected in all microtubule subpopulations. In the microtubules of flagella, acetylation was detectable during axoneme polymerization, indicating the absence of a lag between assembly and modification (Sasse & Gull, 1988). The same authors, however, demonstrated the presence of acetylated α-tubulin in the ephemeral intranuclear spindle microtubules, suggesting that there was no correlation between occurrence of acetylated α-tubulin and microtubule stability (Sasse & Gull, 1988). Studies carried out in *Chlamydomonas* (Le Dizet & Piperno, 1986) and *Physarum polycephalum* (Diggins & Dove, 1987; Sasse et al., 1987) showed that a subset of cytoplasmic microtubules found in these organisms also contains acetylated α-tubulin. However, these microtubules are not so abundant as the sub-pellicular microtubules of trypanosomatids, making the latter organisms a suitable model for further studies on the mechanism of acetylation of α-tubulin and its influence on the functional role played by the microtubules.

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