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PLASMA MEMBRANE VESICLES ISOLATED FROM EPIMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI*

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Summary

Membrane vesicles can be obtained from epimastigote forms of *Trypanosoma cruzi* by incubating cells with either cross-linking reagents or acid pH. Acetate, phtalate or citrate, at pH 4.0, but not at higher pH values, were able to induce plasma membrane vesiculation. Vesicles have been purified by sucrose density centrifugation and their membrane origin was demonstrated by the following criteria: (a) Vesicles are 5—10 times richer in protein-bound iodine when they are prepared from cells previously labeled with ^{131}I by the lactoperoxidase catalysed reaction. (b) Electron microscopy of vesiculating cells shows physical continuity between cell plasma membrane and vesicle membrane. (c) Antibodies prepared against purified vesicles are able to agglutinate epimastigote forms of *T. cruzi* with sera dilutions up to 1 : 256 to 1 : 512. (d) Freeze-fracture studies of the purified vesicles have shown images of faces P and E compatible with known images of the intact cell plasma membrane.

Typical preparations of acetate vesicles present the following characteristics: total carbohydrate : protein = 1.5—2.0; orcinol : protein = 0.07 and absence of diphenylamine reaction. Vesicles contain 0.2—0.5% and 0.3—1.0% of the total homogenate protein and carbohydrate, respectively. The presence of 10 major protein bands and a 30—50-fold enrichment of the four sugar-containing macromolecules present in epimastigote forms of *T. cruzi* have been demonstrated in these preparations.

Introduction

The biological cycle of *Trypanosoma cruzi*, a digenetic parasitic protozoon and the causative agent of Chagas' disease [1,2], shows important feature as

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yet uncovered, namely the nature of the signals which determine transformation of a given cell type into the other, the mechanisms involved in the interaction between parasite and vertebrate host cells and, finally, the nature of the immune response [2,3].

These features depend both on receptor-ligand interactions and on cell recognition and constitute important membrane-dependent phenomena in several cell systems studied to date [4—6].

Thus, knowledge of the chemical composition and topography of *T. cruzi* plasma membrane is a fundamental step to the understanding of the biological problems involved in the evolutive cycle of this protozoon.

Several cells and erythrocyte ghosts shed fragments (vesicles) from their surface membranes to the outside environment, when subjected to specific treatments [7—16]. These treatments yield fairly pure membrane fragments enriched in certain components [17—19] allowing a reasonably precise analysis of the plasma membrane topography [20,21].

In the present work a method of isolation of plasma membrane vesicles from *T. cruzi* epimastigotes along with a general analysis of their properties are described.

Materials and Methods

Cell cultures. Epimastigote forms of *T. cruzi*, Y strain [22], were cultured at 28°C in a rotatory shaker (New Brunswick) at 120 rev./min. Cells in late exponential growth (80—100 h) were harvested by centrifugation at 1000 × *g*, 4°C, and washed 3 times with 150 mM NaCl. Cell numbers were determined in a Coulter counter.

Vesicle isolation. $1 \cdot 10^9$—$5 \cdot 10^9$ cells/ml were incubated at 37°C for up to 60 min with the vesiculating agent (see legends to the figures and tables) dissolved in a solution of 150 mM NaCl/1 mM $CaCl_2$/0.02% NaN_3. After incubation the cells were centrifuged at 4000 × *g* for 10 min at 4°C and the supernatant was collected. The cell pellet was washed twice with cold 150 mM NaCl (1 ml per $1 \cdot 10^9$—$2 \cdot 10^9$ cells) as described above and the vesicles containing supernatants were pooled. Vesicles were then precipitated at 100 000 × *g* for 1 h at 4°C. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and was further purified in a discontinuous sucrose gradient in order to eliminate contaminating cells and cell debris. The gradients were performed in Spinco SW27 rotor tubes by sequentially layering 5 ml each of the following sucrose solutions in 10 mM Tris-HCl, pH 7.5: 40%, 35%, 30%, 25%, 20% and 8%. After layering 4—5 ml of a vesicle suspension (0.2—1.4 mg protein) the preparation was centrifuged at 16 000 rev./min in the SW-27 Spinco rotor for 16—18 h at 4°C. Fractions of 1 ml were collected from the top with the help of a peristaltic pump. The fractions containing the vesicles (tubes 10—20) were washed twice by centrifugation at 105 000 × *g* with a large excess of 1 mM Tris-HCl, pH 7.5/1 mM EDTA. The preparation was kept at —20°C in 10 mM Tris-HCl, pH 7.5, at a protein concentration of 1 mg/ml.

Lactoperoxidase-catalyzed radioiodination. Washed cells were incubated in 0.1 M phosphate buffer, pH 7.4 ($0.7 \cdot 10^8$—$1.2 \cdot 10^8$ cells/ml) containing

150 mM NaCl, 50 μg/ml lactoperoxidase, 10—20 μM KI and 12—45 μCi/ml of $Na^{131}I$. H_2O_2 was added in four steps at 1.5-min intervals in amounts equivalent to 8 μM final concentration. H_2O_2 concentration was estimated before each experiment by the absorbance at 230 nm ($\epsilon = 72.4\ l \cdot mol^{-1} \cdot cm^{-1}$) [23]. Incubation was carried out at 30°C for 6 min. After incubation cells were washed with large volumes of 150 mM NaCl and then added to non-labeled cells before vesiculation. Radioactivity incorporated into cells and vesicles was determined after two precipitations with cold 7% trichloroacetic acid. The precipitates were resuspended in 0.5 ml 0.5 M NaOH and counted in a Nuclear Chicago gamma counter at an efficiency of 40%. The results shown were not corrected for such an efficiency.

The iodination procedure did not impair cells as judged by their normal motility under phase contrast microscope. The estimated iodination efficiency was 0.3—1.5%. Omission of lactoperoxidase and/or H_2O_2 decreased iodine incorporation to 1.0—2.0% of the maximum.

$Na^{131}I$ was obtained from the Instituto de Energia Atômica, São Paulo, and the lactoperoxidase was prepared as previously described [24].

Analytical procedure. Protein was estimated by the method of Lowry et al. [25] using bovine serum albumin as standard. Nucleic acids were extracted as described [26]. DNA was measured by the diphenylamine reaction modified by Giles and Myers [27] and RNA by the orcinol method [28]. Total carbohydrate was measured by the phenol-sulfuric acid reaction [29] in trichloroacetic acid-precipitated vesicle material after extensive washing and dialysis against 10 mM Tris-HCl, pH 7.5. Sugar-containing macromolecules were analysed by electrophoresis in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as previously described [30]. Proteins were analysed in 10% gels in the presence of 0.1% sodium dodecyl sulfate [31]. For protein analysis cell homogenates and vesicles were previously extracted twice with chloroform/methanol (2 : 1, v/v), twice with *n*-butanol, dialyzed against 1 mM EDTA followed by H_2O, and lyophilized. Gels were stained with periodic acid-Schiff for carbohydrates [32] and Coomassie Blue for proteins [33].

Antibody preparation. Rabbits were injected with vesicles obtained from cells treated with 200 mM acetate buffer, pH 4.0, in the presence of 150 mM NaCl and 1 mM $CaCl_2$ for 30 min at 37°C. Animals were weekly injected with 0.3 mg vesicle protein in 0.1 ml 150 mM NaCl. In the first week an equal volume of Freund's complete adjuvant was injected with the antigen. In the following 4 weeks the latter was replaced by Freund's incomplete adjuvant. In order to test for antibody production 10 μl of appropriately diluted serum, previously inactivated at 56°C for 60 min, were added to 10^6 cells in 10 μl 150 mM NaCl placed in glass slide wells. Agglutination was followed under phase contrast microscope after 15 min incubation at room temperature. Controls were made with 1 : 10 dilutions of rabbit sera collected before antigen injection.

Electron microscopy. Pellets from both cells and vesicles were fixed in 2% (w/v) redistilled glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.2, for 2 h at 4°C. Fixed pellets were washed overnight with phosphate buffer and post-fixed in 1% (w/v) OsO_4 in phosphate buffer for 2 h at 4°C. The pellets were then dehydrated in a graded series of ethanol and embedded in Araldite.

Ultrathin sections made with a Sorvall MT-2 ultramicrotome were stained with uranyl acetate [34] and lead citrate [35].

For freeze-cleavage studies the glutaraldehyde-fixed pellets were glycerinated by sequential immersion in 10 and 20% glycerol solutions containing 0.9% NaCl. After quick-freezing in 'Freon 22' (—155°C) the pellets were fractured in a Balzers (BAF 301) freeze-fracture apparatus. Ultrathin sections and replicas of freeze-cleaved material were photographed in a Zeiss EM9A and in a Phillips 301 electron microscope.

Results

Vesiculating agents. Incubation of *T. cruzi* epimastigote forms with aldehydes, *N*-ethylmaleimide, *p*-chloromercuribenzoate and acid buffers induces cell vesiculation (Table I). This phenomenon, which can be followed under phase contrast microscope, is followed by liberation of the vesicles to the extracellular medium. Alkaline solutions do not induce vesiculation under the conditions used.

Vesicle purification. The $4000 \times g$ supernatants from cells subjected to vesiculation are very rich in vesicles but contain some intact cells and cell debris. This material yields two main protein bands when centrifuged in a discontinuous sucrose gradient (Fig. 1) as described in Materials and Methods. The band corresponding to gradient fractions 10—20 contains pure vesicles. Vesicles concentration diminishes abruptly from fraction 21 to the top of the gradient. These fractions contain all remaining cells and debris. When fractions

TABLE I

CONDITIONS FOR *T. CRUZI* PLASMA MEMBRANE VESICULATION

10^9 cells/ml were incubated at 37°C in the presence of 150 mM NaCl and 1 mM $CaCl_2$. Vesiculation was followed under phase contrast microscope. For the negative results the maximum observation period was 60 min. Citrate, phtalate, acetate, *N*-ethylmaleimide and *p*-chloromercuribenzoate at pH 7.0 were in the presence of 10 mM Tris, pH 7.5.

Agent	Concentration (mM)	pH	Vesiculation
Tris-HCl	10	7.5	—
	200	7.5	—
Sodium phosphate	10	7.0	—
	200	7.0	—
Sodium citrate	200	7.0	—
	200	4.0	+
Sodium phtalate	200	7.0	—
	200	4.0	+
Sodium acetate	200	7.0	—
	200	4.0	+
N-ethylmaleimide	5	7.0	+
	10	7.0	+
p-Chloromercuribenzoate	1	7.0	+
Formaldehyde	10	3.5	+
	250	3.5	+
	250	7.0	+
Acetaldehyde	250	4.0	+
	250	7.0	+

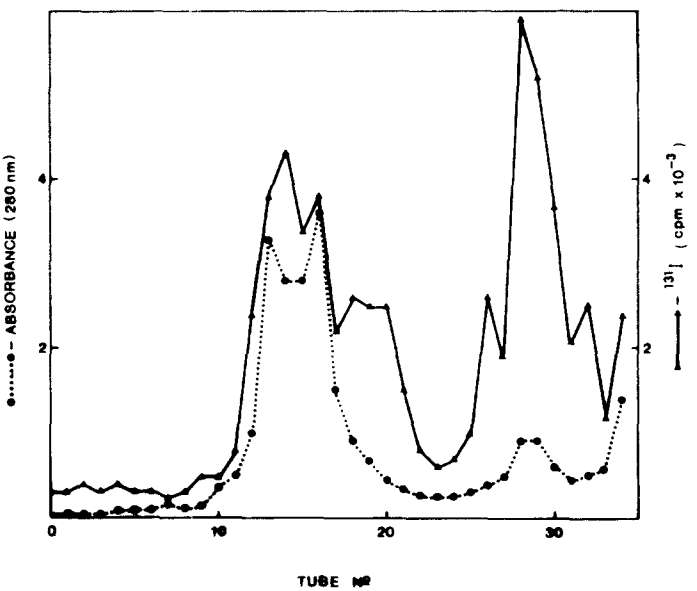


Fig. 1. Purification of *T. cruzi* plasma membrane vesicles by discontinuous sucrose gradients. Cells (10^9) were incubated with lactoperoxidase and $Na^{131}I$ (0.36 mCi) as described in Materials and Methods. These cells were washed and added to a suspension containing $8.8 \cdot 10^{10}$ non-labeled cells. The suspension was then incubated with 200 mM acetate buffer, pH 4.0, for 30 min at 37°C in the presence of 150 mM NaCl and 1 mM $CaCl_2$ and vesicles were subjected to gradient centrifugation as described in Materials and Methods. After centrifugation 1-ml fractions were collected and the absorbance at 280 nm was determined. To each fraction 3.6 mg of bovine serum albumin were added followed by trichloroacetic acid to a final concentration of 7%. The pellets were resuspended in 0.5 ml 0.5 M NaOH for radioactivity determinations. The bottom of the gradient is on the left of the figure.

10—20 are pooled, concentrated and recentrifuged in a similar gradient only one peak is observed (not shown). If cells are labeled with radioactive iodine prior to incubation with the vesiculating agent, the specific radioactivity (relative to protein) of vesicles ($0.08 \cdot 10^6$—$2.6 \cdot 10^6$ cpm/mg) is 5—10 times higher than that of total cell homogenate ($0.012 \cdot 10^6$—$0.26 \cdot 10^6$ cpm/mg). It is interesting to note (Fig. 1) that the radioactivity : protein ratio is higher in the gradient region where contaminating cells band compared to the vesicle-rich region. This suggests that vesicles contain a relatively small number of exposed tyrosine and/or histidine residues as compared to intact cells.

Based on the recovery of labeled iodine and total protein it is possible to show that treatment of vesiculated cells with Lubrol (final concentration, 0.001—0.01%), vortex mixing (30 s) or passage through a hypodermic needle does not increase the yield of vesicle recovery.

Vesiculation induced by acid buffers. All acid buffers tested (200 mM, pH 4.0) induce vesiculation. (Table II). The anions acetate, citrate and phtalate (200 mM) were not effective at pH 7.0 (Table I). In a typical preparation vesicles obtained from cells treated with acetate (200 mM, pH 4.0) at 37°C for 30 min show the following characteristics: total carbohydrate : protein = 1.5—2.0, orcinol : protein = 0.07 and absence of diphenylamine reaction. On the average, vesicles contain 1.2—4.7% of the initially bound iodine and 0.2—0.5% and 0.3—1.0% of the total cell protein and carbohydrate, respectively.

TABLE II

INFLUENCE OF pH AND BUFFER SPECIES IN VESICULATION AS MEASURED BY PROTEIN AND BOUND IODINE RECOVERY

$1.7 \cdot 10^9$ cells were incubated with lactoperoxidase and $Na^{131}I$ (0.62 mCi) as in Materials and Methods. Labeled cells were washed and added to a suspension of $18 \cdot 10^{10}$ non-labeled cells. After mixing $3 \cdot 10^{10}$ cells, aliquots were incubated with the indicated buffers at 37°C for 30 min. Vesicles were isolated as in Materials and Methods. One aliquot (control) was incubated with sodium phosphate, pH 7.0. In all experiments the buffer concentration was 200 mM in the presence of 150 mM NaCl and 1 mM $CaCl_2$. Protein and iodine recovery were calculated taking the intact cell values as 100%.

Treatment	Protein recovery (%)	Vesicle-bound ^{131}I	
		cpm	% recovery
Sodium acetate			
pH 4.0	0.19	12 803	1.22
pH 5.0	0.11	6 740	0.64
pH 5.7	0.05	4 510	0.43
Sodium citrate pH 4.0	0.16	11 103	1.05
Sodium phtalate pH 4.0	0.22	25 895	2.46
Sodium phosphate pH 7.0	0	0	0

Factors influencing vesiculation by acetate buffer. Vesicle production increases with time up to 30 min upon cell incubation with 200 mM acetate, pH 4.0, as measured by the recovery of vesicle-bound iodine. Examination of this phenomenon under phase contrast microscope showed that vesiculation starts as soon as acetate is added to the cell suspension. Vesiculation also occurs at 4°C but with appreciably lower yields (Fig. 2). Table II also shows the pH dependence of *T. cruzi* vesiculation. Better yields are always obtained at pH 4.0. Vesiculation depends also on buffer concentration. It does not occur with acetate concentrations below 20 mM. 200 mM acetate is 10 times more effi-

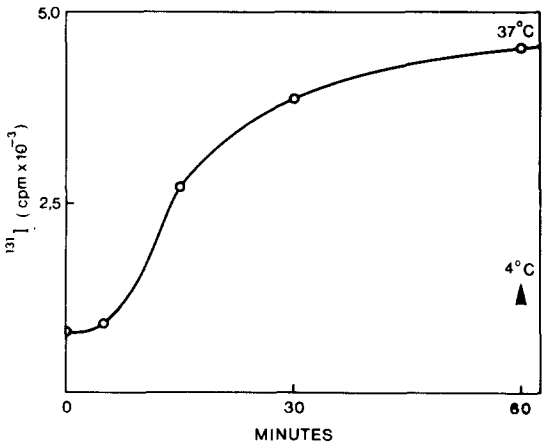


Fig. 2. Effect of the incubation time on the vesiculation induced by acetate buffer. Cells (10^9) were incubated with lactoperoxidase and 0.167 mCi of $Na^{131}I$. After labeling, cells were washed and added to non-labeled cells. Aliquots of $2.1 \cdot 10^{10}$ cells were incubated with 200 mM acetate, pH 4.0 at 37°C (○) or at 4°C (▲) in the presence of 150 mM NaCl and 1 mM $CaCl_2$ for the indicated times. Incubation and vesicle isolation were as described in Materials and Methods.

cient than 20 mM acetate. Similar results, however, are obtained with 200 and 500 mM acetate.

Electron microscopy. Figs. 3–5 show cells incubated with 200 mM acetate, 150 mM NaCl and 1 mM $CaCl_2$, pH 4.0, for 5 min at 37°C. Vesicles are formed from a plasma membrane evagination where the unit membrane can be easily recognized. In general, the forming vesicle appears as a head-like structure and, occasionally, as a narrow protrusion. Vesiculation is observed in the cell body plasma membrane as well as in the flagellar membrane (Fig. 6). Cell organelles are excluded from the vesicles.

Purified vesicles have a homogeneous appearance under the electron microscope with a diameter of approximately 0.5 μm (Figs. 7 and 8). As can be seen, some of the vesicles are made of several overlapped sheets resulting in a denser appearance. This might reflect a tendency to aggregate which is more pronounced if vesicles are frozen and thawed.

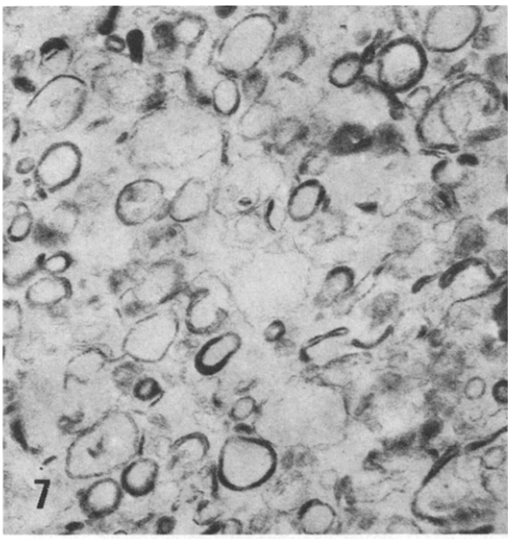
After 30 min incubation with acetate a certain degree of cytoplasm vacuolization may be observed. However, internal organelles are easily identifiable as reasonably intact structures. Vacuolization is intense after 60 min incubation. At this time cells loose approximately 30% of their protein.

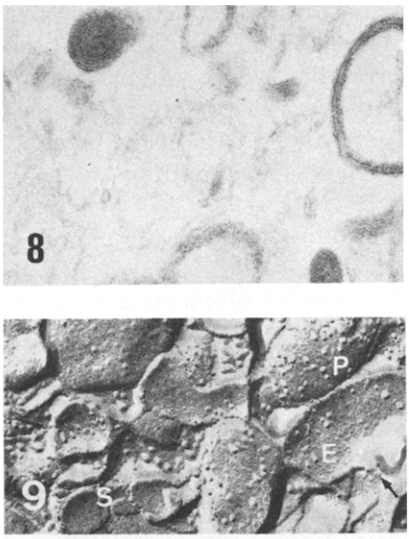
Freeze-fractured vesicles show P and E faces from cleaved membranes; a few smooth membranes (with no particles or with very few particles) were also observed (Fig. 9). Density and diameter of the intramembranous particles are comparable to those found in intact cell membranes [36].

Polyacrylamide gel electrophoresis of the isolated vesicles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the vesicle preparation reveals the presence of about 10 major protein bands as seen by the Coomassie Blue staining (Fig. 10). When gels are stained with periodic acid-Schiff reagent

Figs. 3–5. Plasma membrane evagination (*) and isolated vesicle (↑). Fig. 3, 28 000×; Figs. 4 and 5, 45 000×.

Fig. 6. Plasma membrane evagination in a flagellum. 4500×.





Figs. 7 and 8. Vesicle pellet depicting single membrane and multilamellated vesicles. Fig. 7, 28 500X; Fig. 8, 50 000X.

Fig. 9. P, E and smooth (S) faces of freeze-cleaved isolated vesicle membranes. The arrow indicates the shadowing direction. 50 000X.

(Fig. 10) the typical pattern for *T. cruzi* glycoproteins appear [37]. The faster component is the lipopeptidophosphoglycan previously described [38,39]. The component which migrates with and ahead of the marker dye can be stained (not shown) with the Schiff reagent without previous periodate oxidation, suggesting the presence of reactive carbonyl functions in this component. Densitometric analyses revealed that vesicles are 30—50 times enriched in these sugar-containing substances when compared to the cell homogenate. A precise

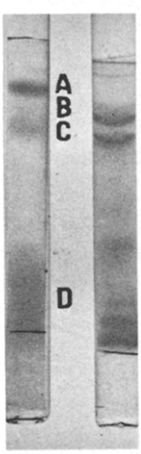


Fig. 10. Polyacrylamide gel electrophoresis of isolated vesicles. The gel on the left was stained with periodic acid-Schiff. The letters refer to sugar-containing substances A, B, C and D. The righthand gel was stained with Coomassie Blue.

quantitative analysis, however, of the composition of the vesicles with respect to these substances will be published elsewhere.

Antibodies. Sera obtained from rabbits previously injected with a vesicle preparation can readily agglutinate suspensions of epimastigote cells up to a dilution of 1 : 512. This result further shows the identity, at least in part, between plasma membrane and vesicles antigens.

Discussion

The vesicles obtained from epimastigote forms of *T. cruzi* upon incubation of the cells with cross-linking reagents or acid buffers are, in all probability, derived from the plasma membrane. This conclusion is drawn from the following experimental results: (1) the specific activity of vesicle-bound radioactive iodine is 5—10 times higher than that found in homogenates prepared from cells previously labeled with ^{131}I by the lactoperoxidase-catalysed reaction; (2) pictures obtained at the electron microscope from vesiculating cells reveal continuity between cell body (or flagellum) plasma membrane and vesicle membrane; (3) vesicles analyzed by the freeze-fracture technique have a general appearance consistent with the plasma membrane topography; (4) antisera prepared against purified vesicles are able to agglutinate intact living trypanosomes.

As in other cellular systems [14,40], vesiculation induced by cross-linking membrane reagents (e.g. aldehydes, *N*-ethylmaleimide and *p*-chloromercuribenzoate) may be due to the binding of these compounds to free sulfhydril or amino groups from plasma membrane proteins.

Acid buffers also induce vesiculation in *T. cruzi*. The pH, rather than the chemical species, seem to be responsible for cell vesiculation since all anions tested are increasingly uneffective as the pH rises. Vesiculation induced by acetate buffer is also dependent on buffer concentration, time and temperature. As it has been found for other cells [14] the formation of surface blebs and vesicles in *T. cruzi* is much more effective at 37°C than at 4°C.

Erythrocyte ghosts are also able to vesiculate at acid pH through an intramembranous aggregation of protein particles, giving as a result protein-depleted vesicles [10,41,42]. That acid-induced *T. cruzi* vesicles are not protein depleted can be inferred from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, taking into consideration that in *T. cruzi* vesicles the carbohydrate : protein ratio is 1.5—2.0 and glycoproteins are 30—50 times more represented than in the total cell homogenate it is possible to conclude that vesicles are relatively richer in sugar-containing substances [37,38] as compared to proteins. This conclusion is apparent if one considers that, in general terms, biological membranes are composed of approximately 10% carbohydrates and 60% proteins [43].

The suggestion can thus be made that either vesiculation takes place at specific membrane regions or that vesiculating compounds increase lateral diffusion of intrinsic membrane macromolecules through the lipid bilayer. Alkaline treatment of sheep erythrocyte ghosts has also been reported to yield glycoprotein-enriched vesicles [19].

If one speculates that glycoproteins A, B and C from *T. cruzi* (Fig. 10 and

ref. 37) are transmembrane macromolecules, as was demonstrated for other membrane glycoproteins from several cell systems [44,45], and that they are somehow associated to the conspicuous net of subpellicular microtubules present in this protozoon [46,47], a suggestion can be made that vesiculating agents induce breakage of these bonds, increasing glycoprotein mobility. This in turn, would result in vesicle formation through membrane destabilization. Nicolson [48] has shown that clustering of sialic acid-containing macromolecules is associated with projections or distortions on the surface of the ghost membrane.

This hypothesis is reinforced by the well known rigidity of the kinetoplastidae plasma membranes [49], attributed to the existence of underlying microtubules [50], and by recent results on the relatively small mobility of concanavalin A receptors in *T. cruzi* epimastigotes (De Souza, W., personal communication). Therefore, *T. cruzi* acetate vesicles might be a convenient material to study particle mobility without possible microtubule interference.

Freeze-fracture studies with the acetate vesicles show the great majority of vesicles rich in intramembranous particles, whereas only a minor proportion is represented by smooth vesicles. The latter are most probably derived from the flagellar membrane since it has been reported that these membranes from *T. cruzi* are almost devoid of intramembranous particles [36].

More work has to be done in order to assess the sidedness of the isolated vesicles. However, the great majority of P faces seen in the freeze-fracture micrographs are convex, suggesting a predominance of right-side-out membranes.

T. cruzi membranes prepared by classical methods of cell fractionation contain an enriched activity of adenylyl cyclase [51]. However, it has not been possible to demonstrate such activity in vesicles. This can be explained by an inactivation of the enzyme during acetate incubation which also occurs under standard assay conditions. A systematic study of vesicles enzyme and protein composition will be published elsewhere.

Finally, it must be said that as the isolated vesicles are antigenic they are a convenient material for immune protection studies during experimental infection.

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