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PURIFICATION OF AN ADENYLYL CYCLASE-CONTAINING PLASMA MEMBRANE FRACTION FROM *TRYPANOSOMA CRUZI*

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Summary

A fraction containing plasma membrane fragments has been purified from epimastigote forms of *Trypanosoma cruzi*. Cells were broken by sonic vibration under well defined conditions and membranes were isolated by differential centrifugation and equilibrium centrifugation in sucrose gradients. The co-purification (approximately 10-fold) of adenylyl cyclase and plasma membrane-bound radioactive iodine is highly suggestive of the localization of this enzyme in the plasma membrane of *T. cruzi*. Determination of succinate cytochrome *c* reductase and glucose-6-phosphatase activities, as well as of total amounts of DNA and RNA in the purified fraction, indicates a negligible contamination from other cellular organelles.

The co-purification of acid phosphatase activity with bound labeled iodine and adenylyl cyclase was taken as circumstantial evidence that part of this enzyme also belongs to the plasma membrane of *T. cruzi*.

Conventional electron microscopy and freeze-fracture images of this fraction are consistent with a highly enriched plasma membrane preparation.

Introduction

The few attempts that have been made to isolate plasma membrane fragments from trypanosomatids encountered some difficulties derived from (a) the rigid subpellicular skeleton of microtubules making the organism quite resistant to the conventional cell breakage methods [1,2] and (b) the existence of a single large mitochondrion per cell [3,4] yielding fragments of different sizes when drastic cell lysing methods are applied. These peculiar characteristics

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complicate the separation of activities belonging to different organelles.

Considering that both the immune response elicited by infection and the penetration of this pathogenic protozoon into host cells most probably involve membrane recognition and interaction it becomes important to obtain fairly pure plasma membrane fragments from *Trypanosoma cruzi*. Analysis of similarities and differences in the chemical make up of the plasma membrane from the three main cell forms found in the parasite's life cycle [5] might throw some light on these fundamental problems.

Furthermore, external signal reception and amplification have been attributed to plasma membrane components in other cell systems studied to date [6]. Some of these components, like the adenylyl cyclase, are thought to be involved in the differentiative transition [7,8]. In *T. cruzi*, where the kinetic properties of this particulate enzyme have already been reported [9], external modulators might play a role in growth and differentiation through modification of the cyclase activity. Such a possibility makes it mandatory to show the association of this enzyme with the plasma membrane.

In this report a procedure for the purification of an adenylyl cyclase-rich plasma membrane fraction from epimastigote forms of *T. cruzi* is described. The identity and the purity of such membranes were assessed by several independent criteria, biochemical and ultrastructural.

Materials and Methods

Cell culture. Epimastigote forms of *T. cruzi*, Y strain [10] were cultured in LIT medium [11] in a rotatory shaker (New Brunswick) at 120 rev./min, 28°C. Cells (approx. $2.5 \cdot 10^{10}$) were harvested during late exponential growth (90—100 h) by centrifugation at 1000 $\times g$ (4°C). The protozoa were washed 3 times with 300 ml of 0.9% (w/v) NaCl solution.

Cell disruption and fractionation. The washed cellular pellet ($2.5 \cdot 10^{10}$ cells) was resuspended in 4.5 ml 10 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and sonicated for 15 s in a Branson sonifier, model W185D (5-s pulses with 1 min interval between pulses) in glass tubes of 2.3 cm diameter. Sonic pressure corresponded to the displacement of 1.2 g given by the half inch diameter probe half immersed in 50 ml distilled water contained in a 100 ml beaker (4.5 cm diameter $\times$ 6.7 cm height). The homogenate was diluted to 130 ml with the same buffer as above, and fractionated at 4°C at 750 $\times g$ for 10 min (P_1), 5000 $\times g$ for 30 min (P_2), 14 000 $\times g$ for 30 min (P_3) and, finally, at 105 000 $\times g$ for 60 min (P_4). A Sorvall RC-5 was used for the intermediate speed centrifugation and a Spinco L-3 50 model for high speed centrifugation. The pellets were resuspended in 10 mM Tris-HCl, pH 7.5, and stored at —20°C.

Analysis of the enriched membrane fraction by sucrose gradient centrifugation

Lubrol-containing gradients. Samples (8—12 mg protein, 2 ml) were treated for 10 min at 4°C with 0.5% Lubrol PX (w/v) (Sigma), layered on top of gradients, and centrifuged in an SW 27 rotor in the Spinco L-3 50 ultracentrifuge, for 5 h at 26 000 rev./min at 4°C. Linear sucrose gradients (0.1—1.0 M, 28.8 ml total volume) were prepared over two cushions, one of 1.8 ml of 2.0 M sucrose, and the other of 2.5 ml of 1.5 M sucrose. All sucrose solutions used for these gradients contained 10 mM Tris-HCl, pH 7.5, and 0.5% Lubrol PX (w/v).

Sucrose gradients. Samples (8—10 mg protein, 3 ml) were dialyzed for 7 h at 4°C against 10 mM Tris-HCl/1 mM EDTA, pH 7.5 (4 l), and were layered on top of gradients. The tubes were centrifuged in the same rotor as above, for 15 h at 16 000 rev./min at 4°C. The gradients were made by sequentially layering 3 ml 50% sucrose, 5 ml 40% sucrose, 8 ml 35% sucrose, 8 ml 30% sucrose, 5 ml 25% sucrose and 3 ml 20% sucrose. All sucrose solutions contained 10 mM Tris-HCl, pH 7.5. The composition of sucrose solutions is expressed in % (w/v).

In both cases, after centrifugation samples (1 ml) were collected from the bottom of the tube by means of a peristaltic pump. The absorbance at 280 nm was measured in a Zeiss (PMQ II) spectrophotometer. Analyses were performed immediately or else the fractions were stored at 4°C.

Enzymatic radio-iodination of cells and determination of radioactivity. The iodination procedure was adapted from several sources [12,13]. Epimastigote forms of *T. cruzi* washed in 0.9% (w/v) NaCl were incubated (10^8 cells/ml) in 0.1 M sodium phosphate buffer, pH 7.4, containing the following compounds in the final concentrations indicated: 0.15 M NaCl, 10 μM KI, 150 μCi/ml $Na^{131}I$, and 50 μg/ml lactoperoxidase. H_2O_2 was added in four steps, with 1.5 min intervals, in amounts equivalent to 8 μM final concentration. Incubation was performed at 30°C for 6 min. The reaction was stopped by the addition of cold 0.9% (w/v) NaCl, and the cells were immediately centrifuged. The organisms were washed three times in 5 vols. of the above solution in order to remove unchanged ^{131}I and lactoperoxidase, and then added to non-labeled cells. Cell disruption and fractionation was performed as above. To ensure that all radioactivity measured was covalently bound to protein, all samples were precipitated twice with cold 7% (w/v) trichloroacetic acid before being dissolved in 0.5 ml of 1 M NaOH and counted in a Nuclear Chicago gamma counter at an efficiency of 40%. The gradient fractions were precipitated as above after the addition of bovine serum albumin (1 mg/ml) as carrier. 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.

Solutions of H_2O_2 were prepared immediately before use by dilution of a stock solution in phosphate buffer. The concentration of H_2O_2 was determined spectrophotometrically at 230 nm ($\epsilon = 72.4\ l \cdot mol^{-1} \cdot cm^{-1}$). Lactoperoxidase was purified as described [14]. The enzyme concentration was determined spectrophotometrically at 412 nm, assuming $\epsilon = 11.4 \cdot 10^4\ l \cdot mol^{-1} \cdot cm^{-1}$. $Na^{131}I$ was obtained from the Instituto de Energia Atômica, São Paulo.

Enzymatic assays. Adenylyl cyclase (EC 4.6.1.1) was assayed under the conditions described by Franco da Silveira et al. [9]. The enzymatic activity is expressed as nmol cyclic AMP/min per mg protein, at 30°C.

Succinate cytochrome *c* reductase (EC 1.3.99.1) was assayed exactly as described by Sottocasa et al. [15] except that the reaction volume was 1 ml instead of 3 ml. The activity is expressed as nmol cytochrome *c* reduced/min per mg protein at 25°C.

Acid phosphatase (EC 3.1.3.2) was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (10^{-3} M) in the presence of 50 mM acetate buffer/0.5 mM EDTA, pH 5.0. The activity is expressed as nmol *p*-nitrophenol/min per mg protein at 37°C.

Glucose-6-phosphatase (EC 3.1.3.9) was assayed as described [16]. Activity was measured in the presence of 2 mM EDTA to inhibit alkaline phosphatase and 2 mM NaF to inhibit acid phosphatase. Inorganic phosphate was determined by the method of Ames [17]. The enzymatic activity is expressed as nmol inorganic phosphate liberated from glucose-6-phosphate/min per mg protein at 37°C.

Analytical procedures. Protein was measured by the method of Lowry et al. [18] using bovine serum albumin as standard. Nucleic acids were extracted according to Schmidt-Thannhauser [19]. DNA was measured by the diphenylamine reaction modified by Giles and Myers [20], using calf thymus DNA as standard. RNA was determined by the orcinol reaction [21] using yeast RNA at standard.

Electron microscopy. The precipitates of the various cellular fractions were fixed in 2% (w/v) redistilled glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.2, for 2 h at 4°C. The fixed pellets were washed several times with the phosphate buffer and post-fixed in 2% (w/v) buffered OsO_4 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 [22] and lead citrate [23]. 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 materials were photographed in a Zeiss EM9A and in a Philips 301 electron microscope.

Results

Distribution of adenylyl cyclase in cellular fractions of T. cruzi. Epimastigote forms of *T. cruzi* were disrupted by sonication in 10 mM Tris-HCl/10 mM $MgCl_2$, pH 7.5, and fractionated by differential centrifugation, as described in Materials and Methods. Adenylyl cyclase activity was found to be 5—6 times greater in the 14 000 $\times g$ fraction as compared to the whole cell homogenate (Table I). This fraction contains 24% of the total enzymatic units.

Distribution of biochemical markers in cellular fractions of T. cruzi. Although the presence of adenylyl cyclase in the particulate fraction of *T. cruzi* cells has been shown before [9] its exact localization remained to be proved. The distribution pattern of this enzyme has been examined in parallel with the distribution of ^{131}I previously bound to the cells by the lactoperoxidase catalyzed reaction [12,13]. Optimal labeling of the cells was obtained with iodide at $8 \cdot 10^{-6}$ M at 30°C for 6 min.

The distribution of ^{131}I incorporated in the cellular fractions of *T. cruzi* can be seen in Table II. From the total incorporated radioactivity 14% is found in the 14 000 $\times g$ fraction, the specific activity being 4 times higher than that found in the whole cell homogenate.

The enzymes chosen as markers for the presence of cellular organelles were succinate cytochrome *c* reductase, acid phosphatase and glucose-6-phosphatase, biochemical markers of mitochondria, lysosomes and microsomes, respectively,

TABLE I

DISTRIBUTION OF ADENYLYL CYCLASE IN CELLULAR FRACTIONS OF *T. CRUZI*

Epimastigote forms of *T. cruzi* were disrupted by sonication in 10 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. Cellular fractionation was performed as described in Materials and Methods. Enzymatic specific activity is expressed as nmol cyclic AMP produced/min per mg protein. Results are expressed as the mean of duplicate determinations of three separate experiments. S_4 is the supernatant from the 105 000 × *g* centrifugation.

Fraction	Protein (%)	Adenylyl cyclase	
		Spec. act.	Total units (%)
Homogenate	100	0.25	100
P_1 (750 × *g*)	30	0.33	42.4
P_2 (5000 × *g*)	10	0.64	27.5
P_3 (14 000 × *g*)	3.8	1.50	24.4
P_4 (105 000 × *g*)	1.5	0.87	5.6
S_4	54.6	0	0

in mammals. The validity of the application of such markers in *T. cruzi* cells will be discussed below.

The data presented in Table II show that the 14 000 × *g* fraction, enriched in adenylyl cyclase and bound iodine, contains only 0.6% of the total succinate cytochrome *c* reductase units and approximately 10% of the total acid phosphatase and glucose-6-phosphatase units.

Determination of the distribution of DNA shows that 98% is concentrated in fraction P_1 and 2% in fraction P_2. On the other hand, 63% of RNA is located in P_1, 8% in P_2 and 2.5% in P_3. The remaining is distributed between P_4 and cytosol.

The data presented lead to the conclusion that the 14 000 × *g* fraction is enriched in plasma membrane fragments with little contamination by other organelles.

TABLE II

DISTRIBUTION OF BIOCHEMICAL MARKERS IN CELLULAR FRACTIONS OF *T. CRUZI*

Epimastigote forms of *T. cruzi* were labeled with ^{131}I and lactoperoxidase and disrupted by sonication in 10 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. Cellular fractionation was performed as described in Material and Methods. Iodine specific radioactivity is expressed as cpm $(\times 10^3) \cdot mg^{-1}$. Enzymatic specific activity is expressed in all cases as nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. The values in parentheses represent the percentage from the total. Results are expressed as the mean of duplicate determinations of two experiments. S_4 is the supernatant from the 105 000 × *g* centrifugation.

Fraction	Protein	Iodine	Succinate cytochrome *c* reductase	Acid phosphatase	Glucose-6-phosphatase
Homogenate	(100)	35.0 (100)	8.8 (100)	6.7 (100)	13.9 (100)
P_1 (750 × *g*)	(30)	57.5 (50)	21.7 (91)	6.7 (30)	27.3 (59)
P_2 (5000 × *g*)	(10)	71.1 (20)	8.3 (6)	14.3 (11)	20.8 (15)
P_3 (14 000 × *g*)	(3.8)	129.1 (14)	1.8 (0.6)	28.7 (11)	36.5 (10)
P_4 (105 000 × *g*)	(1.5)	69.2 (3)	1.3 (1.1)	39.3 (16)	100.6 (12)
S_4	(54.6)	7.1 (11)	0 (0)	4.8 (31)	0 (0)

Distribution of adenylyl cyclase and bound iodine in sucrose gradients containing Lubrol. The observation that in the 14 000 × *g* fraction the specific activities of both adenylyl cyclase and labeled iodine are 4—6 times higher than in the whole cell homogenate lead to the question of whether these two markers were physically linked to the same structure.

Two preparations of *T. cruzi* cells, one labeled with ^{131}I, were processed and fractionated in identical manner. Fraction P_3 (14 000 × *g*), enriched in plasma membrane, was treated with the detergent and centrifuged in sucrose gradients, as described in Materials and Methods. Fig. 1 shows the 280 nm profiles of both preparations. Two peaks, one near the top and the other near the bottom of the gradient, can be distinguished. The latter contains the residual activity of the succinate cytochrome *c* reductase present in the 14 000 × *g* fraction. The distribution of labeled iodine (Fig. 1A) shows two regions, the peak near the bottom of the gradient containing 30% of the total radioactivity, while the remaining 70% is located near the top. Negligible adenylyl cyclase activity can be detected in the lower portion of the tube. The enzyme is concentrated in the top two thirds of the gradient in a region coincident with one of the radioactive iodine peaks. This experiment shows that most of the bound iodine co-migrates with the adenylyl cyclase activity, suggesting their location in the same structure.

Treatment of P_3 fraction with Lubrol PX in a concentration up to 3% (w/v) does not promote solubilization of the *T. cruzi* adenylyl cyclase but inactivates 25—50% of the enzyme. For this reason the use of detergent to purify the membrane fragments was avoided.

Purification of 14 000 × g fraction by sucrose gradient centrifugation. In order to achieve further purification of the plasma membrane present in P_3 fraction other conditions of centrifugation were devised. The 14 000 × *g* fraction was dialyzed against 10 mM Tris-HCl/1 mM EDTA, pH 7.5 in order to eliminate particle-bound Mg^{2+}. Samples, one of them labeled with ^{131}I, were centrifuged in discontinuous sucrose gradients, under the conditions described in Materials and Methods. The radioactive and A_{280nm} profiles obtained are depicted in Fig. 2. Adenylyl cyclase activity can be detected in the 35% (w/v) sucrose layer, concentrated in a well defined peak (Fig. 2B). The migration of this enzyme was coincident with part of the acid phosphatase activity (Fig. 2, A and B) and labeled iodine (Fig. 2A). Another peak containing acid phosphatase and ^{131}I banded in the 30% (w/v) sucrose layer. This peak, however, is considerably deprived in adenylyl cyclase activity. The possible nature of this peak will be discussed later.

Fractions 7—13 from the gradients were independently pooled, diluted with 10 mM Tris-HCl/10 mM $MgCl_2$, pH 7.5, in order to lower the sucrose concentration to 10%, and centrifuged at 80 000 × *g* for 1 h. The pellet obtained (P'_3) was analyzed by electron microscopy and for its content in adenylyl cyclase, acid phosphatase and labeled iodine. The recovery of these three markers in P'_3 was around 95—100% when compared to the pooled gradient fractions. The specific activities of acid phosphatase and radioactive iodine in the pellet are 2.5 times higher than in the 14 000 × *g* fraction. Adenylyl cyclase specific activity is 1.7 times greater than that found in the original P_3 fraction. Probably the sligth differences in enrichment of the three biochemical markers are due to

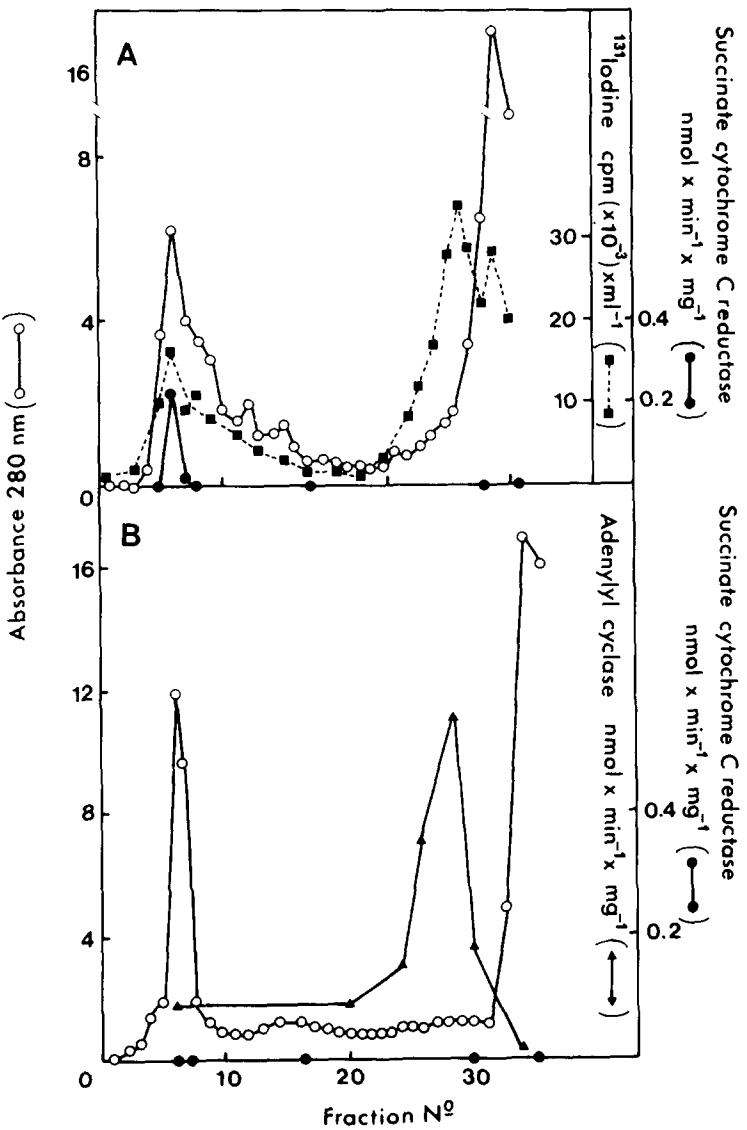


Fig. 1. Centrifugation of 14 000 × *g* (P_3) fraction in Lubrol containing sucrose gradients. P_3 (10 mg) after treatment with 0.5% Lubrol PX (w/v) was centrifuged in sucrose gradients containing the same concentration of the non-ionic detergent as described in Materials and Methods. The A_{280nm} profile, as well as the distribution of adenylyl cyclase, succinate cytochrome *c* reductase and bound iodine are analyzed. The bottom of the gradients are on the left. Specific activities of the markers before gradient centrifugation were: adenylyl cyclase, 0.95 nmol · min^{-1} · mg^{-1}; succinate cytochrome *c* reductase, 1.6 nmol · min^{-1} · mg^{-1}; iodine, 129 000 cpm · mg^{-1}. A and B represent two parallel experiments.

partial inactivation of the cyclase during the whole procedure. On the average the three markers in the final membrane preparation (P'_3) were 10-fold purified when compared with the whole cell homogenate. This purified fraction has been found to contain only 4.5% of the total units of glucose-6-phosphatase, an enzyme that may be considered as a microsomal marker since its specific activ-

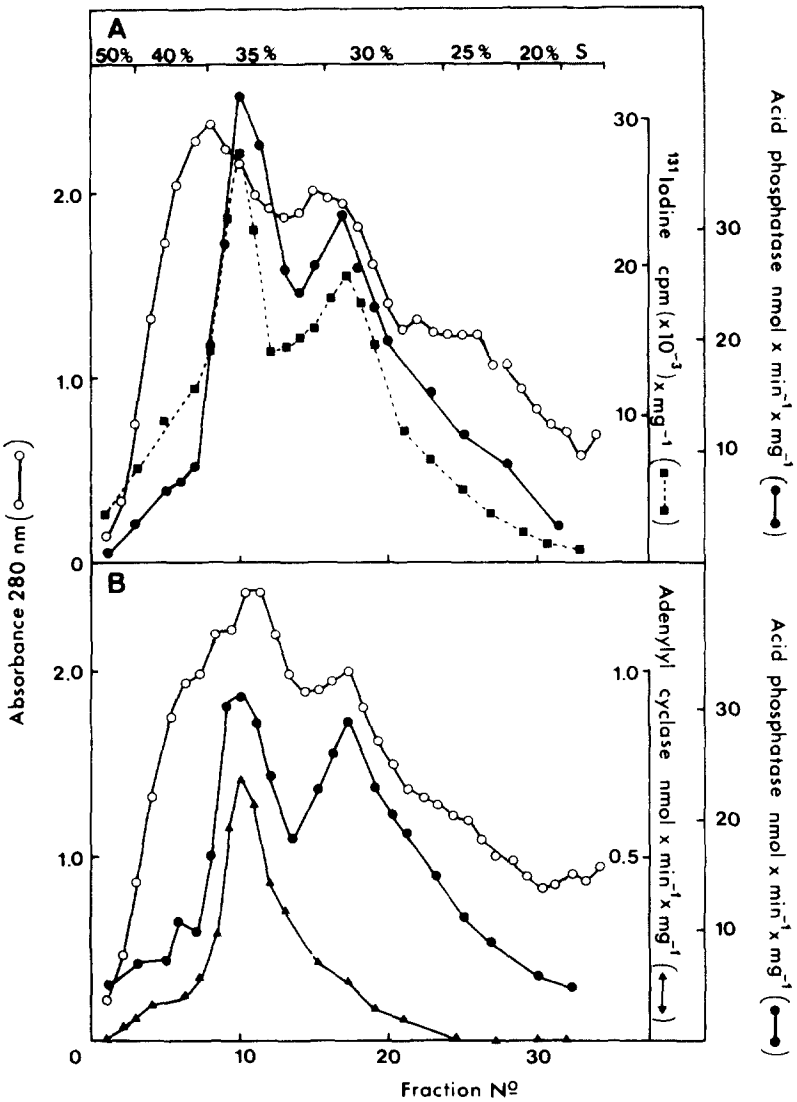


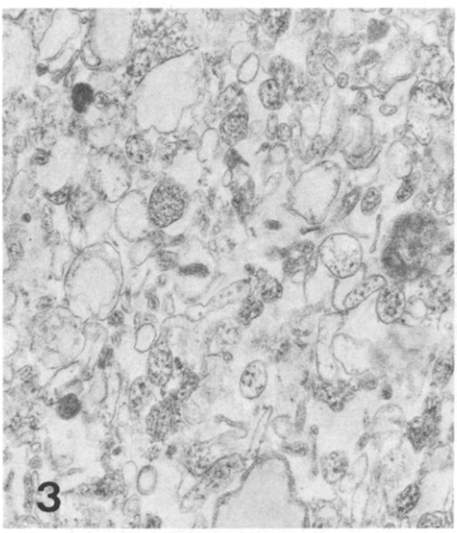
Fig. 2. Centrifugation of 14 000 × *g* (P_3) fraction in sucrose gradients. Fraction P_3 (8 mg), after dialysis, was layered on the top of discontinuous sucrose gradients. Centrifugation was performed as described in Materials and Methods. Distribution of A_{280nm} absorbing material, adenylyl cyclase and acid phosphatase activities, as well as bound ^{131}I were analyzed in the obtained fractions. Initial composition of the sucrose layers are represented on top of the figure. The bottom of the gradients are on the left. Specific activities of the markers before gradient centrifugation were: adenylyl cyclase, 0.39 nmol · min^{-1} · mg^{-1}; acid phosphatase, 19 nmol · min^{-1} · mg^{-1}; iodine, 6500 cpm · mg^{-1}. A and B represent two parallel experiments.

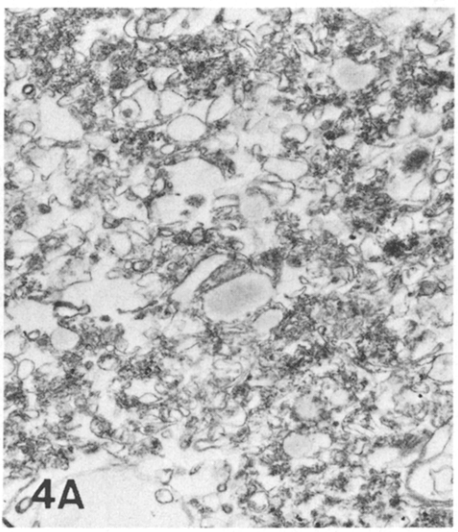
ity is 7.5 times higher in the P_4 fraction as compared to the total cell homogenate (Table II and ref. 28).

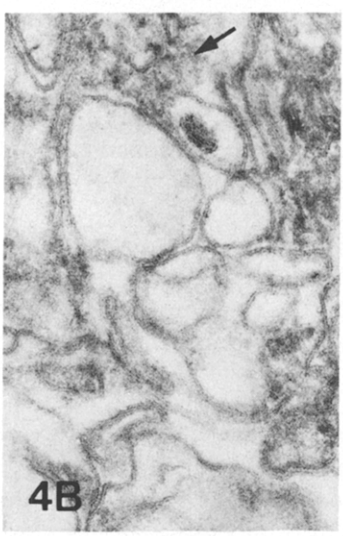
The protein recovered in the pellet represents 1% of the total cellular homogenate and 22% of the 14 000 × *g* fraction.

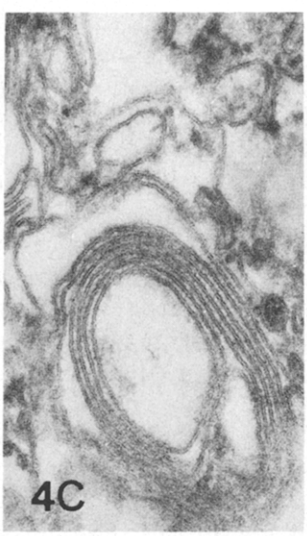
Electron microscopy. Fractions P_1 and P_2 show a highly heterogeneous aspect when analyzed by electron microscopy. In these fractions a great num-

ber of flagella, nuclei, kinetoplasts, mitochondria and ribosomes can be detected (not shown). This observation is confirmed by the distribution of the enzymatic markers employed for the detection of such organelles (Table II). The concentration of the majority of the cellular elements in fractions P_1 and P_2 derives from the technique employed for cell disruption and from the use of









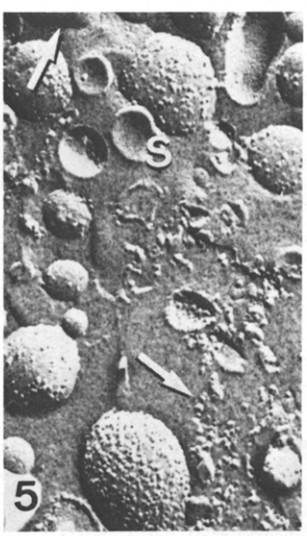


Fig. 3. Partially purified membrane fraction (P_3). (28 500X).

Fig. 4. P'_3 fraction. A, low magnification (14 500X); B, single-membrane vesicles (72 000X); C, multilayered and single-membrane vesicles (72 000X), ↑, amorphous material.

Fig. 5. Freeze-cleaved P'_3 fraction membranes displaying variable densities of intramembranous particles and smooth membranes (S) devoid of particles. ↑, free particles. The arrow in the upper left corner indicates the shadowing direction (55 000X).

Mg^{2+} in the lysing buffer, which permits the formation of aggregates sedimenting at low speed. On the other hand, P_4 (105 000 $\times g$) presents a typical aspect of microsomal fraction, rich in free and bound ribosomes.

P_3 (14 000 $\times g$) shows a certain heterogeneity when analysed under the electron microscope. A great variety of sizes of vesicles can be seen containing some amorphous material plus membrane-enclosed bodies (Fig. 3). The sucrose gradient centrifugation yields a cleaner fraction (P'_3) of membrane vesicles (Fig. 4A) in which a trilaminar appearance is easily visualized (Fig. 4B). These membranes are often seen as multiple layers (Fig. 4C). Size heterogeneity can easily be explained by the method employed for cell breakage. Most of the denser material seen at low magnification (Fig. 4A) has been proved to be collapsed and aggregated plasma membranes usually arranged as myelin figures (Fig. 4B and C). The replicas of freeze-fractured vesicles from P'_3 fraction show cleaved and fractured membranes displaying variable amounts of intramembranous particles and membranes devoid of such particles (Fig. 5). The latter are probably derived from the flagellar membrane [24]. Clumps of globules similar in size to the intramembranous particles can be observed between the membranes. These clumps and the amorphous material seen in the conventional electron micrographs might have the same identity. In other systems, rich in microtubules and microfilaments, amorphous material between purified plasma membrane fragments has been interpreted as fibrils derived from those structures [25,26].

Discussion

In previous attempts to fractionate *T. cruzi* with the aim to localize subcellular antigens [27,28] enzymatic and ultrastructural analyses revealed a reasonable degree of cross-contamination among the several subcellular fractions. The isolation of flagellar and membrane fractions from *T. cruzi* has been recently reported [29]. Unfortunately, the identity of the fractions was only checked by electron microscopy with no reference to the distribution of biochemical markers.

To our knowledge, this is the first report of the isolation of a plasma membrane fraction from *T. cruzi* whose identity has been assessed by a variety of biochemical and ultrastructural criteria. During the preparation of this manuscript another group (Pereira, N.M., personal communication) has been able to achieve the same result using the non-ionic detergent Lubrol during cell rupture.

Plasma membrane fragments from disrupted *T. cruzi* cells sediment mainly at 14 000 $\times g$. This fraction is essentially free from mitochondrial contamination since only 0.6% of the succinate cytochrome *c* reductase total units co-precipitate with P_3. This fraction also contains 10% of glucose-6-phosphatase and 2.5% of the total RNA, indicating the virtual absence of microsomal contamination.

The diversity in the methods employed by several laboratories to disrupt *T. cruzi* is probably the most important explanation for the differences seen in the reported results. We have found that sonication at low power and with short pulses is able to disrupt all cells without excessively damaging internal struc-

tures. Furthermore, the presence of Mg^{2+} in a hypotonic medium seems to promote the formation of aggregates [9] thus facilitating centrifugal separation of non-aggregated material. The distribution of bound iodine (Table II) shows that a large portion of this marker is distributed among other fractions. This means that approximately 80% of the plasma membrane has been lost during purification. However, among the many methods which have been tested this was the only one yielding a fraction rich in plasma membranes and reasonably free from contamination by other organelles.

The results show unequivocally a co-purification of adenylyl cyclase activity and bound radioactive iodine, either in the presence or in the absence of detergent. In gradients made in the absence of detergent the P_3 fraction separates into two protein peaks, one of them being devoid of adenylyl cyclase activity. Since the electron micrographs shown an increased homogeneity in the adenylyl cyclase containing peak as compared to P_3, this second peak banding at 30% sucrose might contain the remainings of contaminant organelles. However, the distribution of bound iodine indicates that part of the plasma membrane fragments also band in that region of the gradient. Plasma membrane fragments from the same organism differing in density and microcomposition have been observed before [25,30—32].

Conventional electron microscopy and freeze-fracture images of the adenylyl cyclase containing peak (P'_3) are consistent with a highly enriched plasma membrane preparation. This evidence, taken together with the co-purification of the enzyme and bound iodine, are highly suggestive that adenylyl cyclase, as in other systems, is a plasma membrane bound enzyme.

Microtubules were absent from the membrane preparations. It is possible that the conditions employed for membrane purification (sonic vibration, low temperature) were responsible for their depolymerization. In *Leptomonas collosoma*, a lower trypanosomatid, Hunt and Ellar [33] have been able to obtain plasma membranes with underlying microtubules. The differences in result might thus be due either to differences in the methods employed or to intrinsic differences in the structural organization of the microtubular net from both organisms.

It must be commented that it has not been possible to find in our preparations 5′-nucleotidase and $(Na^+ + K^+)$-activated ATPase, well established markers of the plasma membrane from mammalian cells. This is in accord with other reports using trypanosomatids (ref. 33 and Pereira, N.M., personal communication) or other cell systems [34,35]. However, caution must be exerted to interpret negative results with the ATPase since it has been recently shown that orthovanadate, a contaminant of most of the commerical ATP preparations, is a potent inhibitor of that enzyme [36].

The general appearance of electron-micrographs and the presence of acid phosphatase comigrating with bound iodine and adenylyl cyclase do not permit the ruling out of a small lysosomal contamination. However, it must be noticed that when P_3 is purified in sucrose gradients the ratios among the activities of bound iodine, adenylyl cyclase and acid phosphatase remain almost constant in the protein peak banding at 35% sucrose. This result could be taken as an indication that in *T. cruzi* acid phosphatase activity is, at least in part, associated with the plasma membrane. This tentative conclusion agrees with sugges-

tions made by several other laboratories [28,33,37].

Another method to prepare plasma membrane vesicles from *T. cruzi* epimastigote cells has been developed [38]. Hopefully, both methods will be useful for the systematic analysis of *T. cruzi* plasma membrane-bound macromolecules.

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References

1 Vickerman, K. (1969) J. Protozool. 16, 54—69
2 Angelopoulos, E. (1970) J. Protozool. 17, 39—51
3 Vickerman, K. (1962) Trans. R. Soc. Trop. Med. Hyg. 56, 487—495
4 Vickerman, K. (1965) Nature 208, 762—766
5 Brener, Z. (1973) Annu. Rev. Microbiol. 27, 347—382
6 Cuatrecasas, P. and Hollenberg, M.D. (1976) Adv. Protein Chem. 30, 251—451
7 Filosa, S., Pictet, R. and Rutter, W.J. (1975) Nature 257, 702—705
8 Vale, V.L., Gomes, S.L., Maia, J.C.C. and Mennucci, L. (1976) FEBS Lett. 67, 189—192
9 Franco da Silveira, J., Zingales, B. and Colli, W. (1977) Biochim. Biophys. Acta 481, 722—733
10 Silva, L.H.P. and Nussenzweig, V. (1953) Folia Clin. Biol. 20, 191—207
11 Castellani, O., Ribeiro, L.V. and Fernandes, J.F. (1967) J. Protozool. 14, 447—451
12 Phillips, D.R. and Morrison, M. (1971) Biochemistry 10, 1766—1771
13 Marchalonis, J.J., Cone, R.E. and Santer, V. (1971) Biochem. J. 124, 921—927
14 Hogg, McC. and Jago, G.R. (1970) Biochem. J. 117, 779—790
15 Sottocasa, G.L, Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415—438
16 Aronson, N.N. Jr. and Touster, O. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. XXXI, pp. 90—102, Academic Press, New York
17 Ames, B.N. (1966) Methods in Enzymology (Neufeld, E.F. and Ginsburg, V., eds.), Vol. VIII, pp. 115—116, Academic Press, New York
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265—275
19 Schmidt, G. and Thannhauser, S.H. (1945) J. Biol. Chem. 161, 83—89
20 Giles, K.W. and Myers, A. (1965) Nature 206, 93
21 Dische, Z. (1955) in The Nucleic Acids (Chargaff, E. and Davidson, J.N., eds.), pp. 285, Academic Press, New York
22 Watson, M.L. (1958) J. Biophys. Biochem. Cytol. 4, 475—478
23 Reynolds, E.S. (1963) J. Cell. Biol. 17, 208—212
24 Martinez-Palomo, A., De Souza, W. and Gonzales-Robles, A. (1976) J. Cell. Biol. 69, 507—513
25 Green, A.A. and Newell, P.C. (1974) Biochem. J. 140, 313—322
26 Heller, M. and Haray, I. (1977) Biochim. Biophys. Acta 467, 29—43
27 Teixeira, A.R.L. and Santos-Buch, C.A. (1974) J. Immunol. 113, 859—869
28 Segura, E.L., Cura, E.N., Paulone, I., Vasquez, C. and Cerisola, J.A. (1974) J. Protozool. 21, 571—574
29 Segura, E.L., Vasquez, C., Bronzina, A., Campos, J.M., Cerisola, J.A. and Gonzales-Cappa, S.M. (1977) J. Protozool. 24, 540—543
30 Evans, W.H. (1970) Biochem. J. 116, 833—842
31 House, P.D.R., Poulis, P. and Weidemann, M.J. (1972) Eur. J. Biochem. 24, 429—437
32 Gilkes, N.R. and Weeks, G. (1977) Biochim. Biophys. Acta 464, 142—156
33 Hunt, R.C. and Ellar, D.J. (1974) Biochim. Biophys. Acta 339, 173—189
34 Schultz, T.M.G. and Thompson, J.E. (1969) Biochim. Biophys. Acta 193, 203—211
35 Ulsamer, A.G., Wright, P.L., Wetzel, M.G. and Korn, E.D. (1971) J. Cell Biol. 51, 193—215
36 Cantley, L.C. Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C. and Guidotti, G. (1977) J. Biol. Chem. 252, 7421—7423
37 Seed, J.R., Byram, J. and Gam, A.A. (1967) J. Protozool. 14, 117—125
38 Franco da Silveira, J., Abrahamsohn, P.A. and Colli, W. (1979) Biochim. Biophys. Acta 550, 222—232