Enrichment of Right-Side-Out *Trypanosoma cruzi* Plasma Membrane Vesicles (42908)

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Abstract. **A simple method to prepare a high yield of *Trypanosoma cruzi* plasma membrane vesicles (PMV) from epimastigotes and metacyclic trypomastigotes is described. The method may be applicable to other protozoa. Solid-phase immunoassay to bind surface *T. cruzi* epitopes showed that this preparation was enriched with 80–82% PMV and that most of these were right-side out (81–92%). The method was based on the extraction of extrinsic proteins and subpellicular tubules with mild high and low ionic strength buffers without detergents (pH 7.4) and on the differential centrifugation of PMV based on their specific density (1.049 g/ml, 4°C). Transmission electron microscopy of PMV pellets showed a heterogeneous population of vesicles without other significant cytoskeletal contaminants. *T. cruzi* PMV were also enriched with an ouabain- and oligomycin-insensitive magnesium-ATPase and contained an adenylyl cyclase, preserved for at least 3 months at −70°C in storage buffer. Measurements of the [^{14}C]-dextran and the $^{3}H_2O$ space indicated that *T. cruzi* PMV were not sealed, explaining why Lubrol PX and NaF failed to stimulate the adenylyl cyclase activity further and why *T. cruzi* PMV were unable to concentrate ^{86}Rb in flow dialysis assays. No detectable DNA and RNA was found. The preparation was not capable of removing ^{51}Cr or [^{3}H]glucosamine from live L6 myoblast surfaces in physiologic conditions and acid phosphatase was extracted by this method. The contaminating fraction (18–20% by immunoassay) consisted of endoplasmic reticulum membranes with NADH oxidase activity and of kinetoplast membranes with cytochrome c oxidase and oligomycin sensitive magnesium-ATPase activity. The biologically active *T. cruzi* PMV retained the ability of living forms to trigger the alternate pathway of complement by releasing the Bb activation fragment from human Factor B.** [P.S.E.B.M. 1989, Vol 191]

Trypanosoma cruzi, the protozoan hemoflagellate implicated in a common but severe form of heart disease, chronic Chagas myocarditis, characteristically parasitizes striated muscle Type I fibers preferentially (1). *T. cruzi* has an obligatory intracellular stage and does not multiply in the blood. The infection is initiated by the fortuitous inoculation of fecal material containing metacyclic trypomastigotes present in the hindgut of the vector, the triatoma insect. American trypanosomiasis occurs rarely in the southwestern tiers of North America but most infected subjects are found in Central and South America. It is estimated that more than 45 million persons are at risk of infection and that 15–25% of infected subjects will develop clinical myocarditis about 10 years following the initial infection (2).

The molecular basis of penetration following maximum *T. cruzi* to host cell contact is unknown and the study of the *T. cruzi*-host surface membrane microenvironment in the initial stage of parasitosis had been previously hampered by the lack of a reliable method to enrich preparations of PMV largely devoid of potential cell-binding contaminants from other components of the parasite cytoskeleton. We have successfully used these preparations to study parasite to host cell interactions in the absence of flagellated movement (3). This *T. cruzi* PMV method was developed for host-tissue infective metacyclic trypomastigotes and for liquid media-grown epimastigotes. In this report, we detail the method for the preparation of enriched right-side out *T. cruzi* PMV and we characterize some of their biochemical and physical properties.

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Materials and Methods

Trypanosoma cruzi. The myotropic Brasil strain of *T. cruzi* was obtained from Dr. Murray Wittner (Yeshiva University, Albert Einstein Medical College, New York, NY). The epimastigote form of the parasite was cultured in liver infusion-tryptose medium containing 10% (v/v) fetal bovine serum (HyClone, Logan,

UT) and gentamicin (Sigma, St. Louis, MO) at 50 μg/ml in 150-cm^2 flasks (Falcon, Oxnard, CA) at 28°C (3). Trypomastigotes were harvested from the liquid medium described by Sadigursky and Brodskyn (4) when more than 90% of the flagellates showed metacyclic forms by direct counts of Giemsa-stained smears. Sadigursky and Brodskyn showed that the infectivity rate of these metacyclic forms was not different from that obtained from blood trypanosomes of infected rats (4).

***T. cruzi* PMV.** *T. cruzi* PMVs were prepared by important modifications of the Jones *et al.* procedure (5) which, after high and low ionic strength extractions, was adjusted to enrich the PMV at their specific gravity (1.049, 4°C). Enrichment of *T. cruzi* plasma membranes had been shown when total homogenates were centrifuged at 14,000*g* in a previous investigation (6). *T. cruzi* flagellates were harvested by centrifugation and washed three times in Hanks' balanced salts solution (Grand Island Biological Co., Grand Island, NY). The pellet (P_1) was resuspended at 20% (v/v) in 0.75 *M* KCl, 10 m*M* histidine (pH 7.4), and stored frozen at −70°C. We have found empirically that higher yields of enzyme markers were obtained when all homogenization procedures were done when the membranes were kept in a slush by lowering the ice bath temperature with rock salt. The thawed suspension (TH) was homogenized by repeated bursts with a Polytron homogenizer (model PT 10204900; Brinkman Instruments, Westbury, NY) (five 5-sec bursts at one half maximal speed) in a rock salt-ice bath and centrifuged at 14,000 *g* for 20 min at 4°C. The pellet (P_2) was resuspended at 10% (v/v) in the KCl-histidine buffer and homogenized by hand with a Potter-Elvheim tissue grinder in a rock salt-ice bath. The suspension was centrifuged at 14,000 *g* for 20 min at 4°C (P_3) and resuspended at 10% (v/v) in 10 m*M* $NaHCO_3$/10 m*M* histidine (pH 7.4) with identical hand homogenization. This homogenate was centrifuged again at 14,000*g* for 20 min at 4°C (P_4) and resuspended at 10% (v/v) in the $NaHCO_3$-histidine buffer. The suspension (P_4) was mechanically homogenized in a Potter-Elvheim tissue grinder (T-line lab stirrer; Talboys, Emerson, NJ) set at 7500 rpm for four 30-sec bursts in a rock salt-ice bath. The homogenate was then centrifuged at 14,000*g* for 20 min at 4°C (P_5).

The supernatant of this centrifugation (S_5) contained the plasma membranes and was centrifuged at 45,000*g* for 30 min at 4°C. The resultant pellet (P_6) consisted of about 75% *T. cruzi* PMV by sialic acid assay which were further purified by resuspension (10%, v/v) in 0.15 *M* sucrose, 0.3 *M* KCl, 0.1 *M* Tris, 50 m*M* sodium pyrophosphate, 0.1% (w/v) bovine serum albumin, or 0.05% (w/v) gelatin (pH 7.4). The enriched PMV suspension was subjected to Potter-Elvheim homogenization (one 30-sec burst, T-line lab stirrer, maximum speed, rock salt-ice bath) and incubated on ice for 30 min. One-half to 1.0 ml of the enriched PMV homogenate was layered over a discontinuous sucrose gradient consisting of a bottom cushion of 2 ml of 0.6 *M* sucrose, 0.3 *M* KCl, 0.1 *M* Tris, 50 m*M* sodium pyrophosphate (pH 7.4), and an overlay of 2 ml of 0.295 *M* sucrose, 0.3 *M* KCl, 0.1 *M* Tris, 50 m*M* sodium pyrophosphate (pH 7.4). The gradient was centrifuged at 30,000*g* for 30 min at 4°C. The purified PMV band was collected from the gradient interface (specific gravity, 1.049; 4°C) and diluted five times with ice-cold distilled H_2O and centrifuged at 45,000 *g* for 30 min at 4°C. The resulting pellet was resuspended at 10% (v/v) in 0.29 *M* sucrose, 0.02 *M* histidine (pH 7.4), and stored frozen at −70°C.

Protein Determination. The Lowry method of protein determination was used with bovine serum albumin (Sigma) as the standard (7).

Isotope Labeling. The surface glycoconjugates of live *T. cruzi* flagellates were labeled with tritium as previously described (3). *T. cruzi* flagellates were also metabolically labeled by the addition of one of the following radiolabeled compounds to the culture medium 5 days before harvesting: L-[^{35}S]methionine, 5×10^{-3} mCi/ml, 500 mCi/mmol (Amersham, Arlington Heights, IL); [*methyl*-^{3}H]thymidine, 5×10^{-3} mCi/ml, 60 mCi/mmol (ICN, Irvine, CA); and [5-^{3}H]uridine, 5×10^{-3} mCi/ml, 30 mCi/mmol (ICN).

Determination of T. cruzi PMV Density. PMV were prepared from tritium surface glycoconjugate-labeled flagellates with the exception that at P_4, 0.5 ml of this suspension was added to a tube containing 10 ml of Percoll (Pharmacia, Piscataway, NJ) in 0.15 *M* NaCl with 15 μl of each of Density Marker Beads 1–9 (Pharmacia) (8). The tube was mixed vigorously and centrifuged for 90 min at 30,000*g*, 4°C. Fractions were collected (0.2 ml each) from the bottom of the tube and counted for radioactivity.

Electron Microscopy. *T. cruzi* PMV pellets (P_6) were fixed in 2% glutaraldehyde-phosphate buffer (pH 7.4) for 1 hr in ice according to established technique. The pellet was postfixed in 1% osmium phosphate and processed without altering the orientation to view cross-sections of the top, middle, and bottom of the pellet. Silver sections were cut, counter-stained with uranyl acetate, and examined at 60 or 80 kV in a JEOL 100C electron microscope.

Preparation of Antisera. Rabbits were immunized with *T. cruzi* PMV as detailed in a previous publication (3). Monospecific IgG anti *T. cruzi* surface antigen (IgG α *T. cruzi* SAg) and its F(ab)$_2$ fragments were thoroughly characterized and the specificity of this antibody was determined in a recently published study (3). Briefly, IgG α *T. cruzi* SAg was rapidly eluted from the surface of live *T. cruzi* epimastigotes with glycine-buffered saline at pH 3 in ice and neutralized. The treatment did not result in elution of *T. cruzi* epitopes as determined by serology and IgG α *T. cruzi* SAg and its F(ab)$_2$ fragment complexed *T. cruzi* PMV in a concentration-dependent fashion (3).

Sidedness by Immunoassay. IgG α *T. cruzi* PMV and IgG α *T. cruzi* SAg each was immobilized on polyacrylamide beads (Affigel; Biorad, Rockville Center, NY) by published technique (9), and suspended at 50% (v/v) in 1% (w/v) bovine serum albumin in phosphate-buffered saline. An equal volume of [^{35}S]methionine-labeled *T. cruzi* PMV (50 μg/ml) was mixed with 200 μl of the antibody-coated bead suspension and incubated at 37°C with gentle agitation for 1 hr. This was followed by an additional 1-hr incubation in ice. The radioactivity of appropriate aliquots of the supernatant and of the twice washed beads was measured. The radioactivity of the antibody against all *T. cruzi* PMV epitopes on both sides of the membrane (IgG α *T. cruzi* PMV) precipitated 88–96% of the [^{35}S]methionine-labeled vesicles (92.1 ± 4.2%, mean ± SEM, n = 3 separate determinations, each in triplicate).

Sidedness by Sialic Acid Assay. One-hundred micrograms of PMV in 50 μl of saline in 0.05 *m* acetate buffer (pH 5.7) was added to 50 μl of neuraminidase immobilized on beads in the presence and in the absence of 0.2% (v/v) Triton X and incubated for 1 hr at 37°C with gentle agitation according to the procedure recommended by the manufacturer (Sigma, St. Louis, MO). The reaction was terminated when the sample was brought to 0.2 ml with distilled water and centrifuged for 30 min at 45,000 *g*, 4°C. Sialic acid chromophores were determined according to the method of Warren (10).

Enzyme Activity Determinations. ATPase activity was determined in a final volume of 1 ml with 50 μg of *T. cruzi* PMV in a basal reaction solution buffered with 20 m*M* histidine (pH 7.4) with additions as indicated and made isoosmolar to plasma with sucrose (311 mOsm/liter). The reaction mixture was equilibrated for 10 min at 37°C and the reaction was initiated by the addition of Tris-ATP to a final concentration of 1.0 m*M*. The reaction was halted after 15 min by the addition of trichloroacetic acid to a final concentration of 5% (w/v). Zero time controls were made by the addition of trichloroacetic acid before the initiation of the reaction and were subtracted. Inorganic phosphate released was determined according to the Marsh procedure (11).

NADH oxidase activity was measured using 50 μg of PMV added to a reaction mixture containing 10 m*M* NADH (Sigma), 30 m*M* NaCN, and the appropriate electron acceptor in 0.1 *M* phosphate buffer (pH 7.4) containing 50 μ*M* EDTA. The reaction was initiated by the addition of 50 μg of *T. cruzi* PMV and absorbance changes at 340 nm were read every 10 sec. NADH was calculated from an extinction coefficient of 6.22×10^3 (12).

Cytochrome *c* oxidase was measured using 25 μg of *T. cruzi* PMV added to a 3.0-ml reaction mixture containing 0.015 *M* reduced cytochrome *c* (Sigma) in 50 m*M* phosphate buffer (pH 7.4). Initial velocities were calculated graphically using an extinction coefficient of 18.5/m*M* (13).

Acid *p*-nitrophenyl phosphatase was measured using 100–270 μg of membrane added to a 3.0-ml reaction mixture containing 0.2% (v/v) Triton X-100 (v/v) in 50 m*M* sodium acetate (pH 4.8) and 5 m*M* sodium *p*-nitrophenyl phosphate (Sigma), using an extinction coefficient at 1.85×10^4/mole *p*-nitrophenyl phosphate (14).

Adenylyl cyclase activity was measured according to the method of Salomon *et al.* (15) with [α-^{32}P]ATP (ICN) as the substrate and ^{3}H-cyclic AMP (ICN) to calculate the recovery from Dowex-alumina columns. Sodium fluoride stimulation and latent activities were assayed in Lubrol PX (Sigma) 2.5 μg/μg membrane (16).

Proteolytic and Glycolytic Activities. The capability of *T. cruzi* PMVs to remove surface bound ^{51}Cr from *L6* myoblast monolayers in oxygenated Krebs-Ringer's bicarbonate buffer (KRB) was measured using our previously published technique (17). Briefly *L6* myoblasts were cultured to confluency in 24-well Costar plates in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) containing 10% (v/v) fetal bovine serum (FBS, HyClone, Logan, UT) and 50 μg/ml gentamicin (Sigma) at 37°C in a humidified atmosphere of 10% CO_2-90% air. After three washes in KRB, the myoblasts were labeled with 50 μl $Na^{51}CrO_4$ (New England Nuclear, Boston, MA) at 100 μCi/ml in KRB. The assays were performed in quintuplicate. After the addition of 50 μg of *T. cruzi* PMV/well, the percentage of ^{51}Cr released at incubation times up to 360 min at 37°C was calculated by well-established statistical methods (18). Glycolytic activity was measured by the release of [^{3}H]glucosamine-labeled surface glycoproteins of *L6* myoblasts (19). The assay, incubation with PMV, and method of calculation were similar to those of the 51-chromium release assay.

[^{14}C]Dextran and ^3H_2O Space. To measure whether *T. cruzi* PMV were tightly sealed, the [^{14}C] dextran (M_r 70,000) and ^3H_2O space were compared according to the technique of Reijngoud and Tager (20). The assay was done in three different preparations in triplicate. Five-hundred micrograms of *T. cruzi* PMV were suspended in 1 ml of isosmolar 305 m*M* sucrose, 5 m*M* imidazole (pH 7.4) containing ^3H_2O (200,000 cpm/ml) and [^{14}C]dextran (200,000 cpm/ml). After 1 hr of incubation in ice, the vesicles were pelleted at 100,000*g* for 30 min at 4°C. The supernatant was removed and the excess on the walls of the tubes was cleaned with a cotton swab. The pellet was resuspended in 500 ml of water and then frozen and thawed several times. The radioactivity for ^3H_2O and [^{14}C]dextran was measured and the equivalent volumes were calculated.

Uptake of ^{86}Rb by Flow Dialysis. This was done with a double chamber and a fitted Spectrapor 1 dialysis membrane (M_r 600–800 cutoff; Fisher Scientific) which

allowed measurement of the pumped effluate from the bottom atrium as previously described (21).

Alternate Pathway of Complement Activation. Activation of the alternative pathway of complement in fresh human serum by *T. cruzi* PMV was demonstrated by immunoelectrophoresis as follows: To one-tenth of 1 ml of whole human serum from a normal donor was added 0.05 ml of a suspension of the *T. cruzi*, PMV previously adjusted to a protein concentration of 1 mg/ml. The mixture was incubated for 30 min at 37°C, immediately chilled, and made 0.01 *M* in EDTA and electrophoresed in 2% (w/v) agar at 40V/slide for 2 hr. Factor B in the electrophoresed sample was detected with monospecific goat antiserum to human Factor B purchased from Miles Scientific (Naperville, IL). Aliquots of the same serum were simultaneously treated with inulin at 50 mg/ml, an activator of the alternative pathway of complement (positive control) and sterile saline as a negative control. A sample of vesicles in saline was processed identically to demonstrate any Factor B-related antigens recognizable by the antiserum and present in the vesicle preparation.

Results

RSO *T. cruzi* Plasma Membrane Vesicle Preparation. The strategy used for preparation of enriched right-side-out *T. cruzi* PMV was based on the ability to extract the extrinsic proteins attached to the plasma membrane with high ionic strength and low ionic strength buffers according to the extensive studies done of myocardial cell membranes by Jones *et al.* (5). The determination of the density of the *T. cruzi* plasma membrane was made by isopycnic centrifugation in PVP-silica gradients utilizing *in vivo* tritium-labeled surface glycoconjugates. The results showed a narrow band corresponding to the density of 1.049 g/ml for the membranes rich in parasite surface glycoconjugates.

After several trials, an optimized purification scheme was established. The Jones procedure for the enrichment of heart sarcolemma vesicles was modified significantly (5) as indicated in Materials and Methods. Approximately 4×10^{10} flagellates yielded 1 mg of *T. cruzi* PMV and preparations ranging from 1.35 to 14 mg protein were obtained. After extraction with 0.75 *M* KCl and subsequently with 0.01 *M* $NaHCO_3$, the PMV were suspended in 0.3 *M* KCl and 0.05 *M* pyrophosphate to increase surface charges with 0.1% (w/v) bovine serum albumin or 0.05% (w/v) gelatin to prevent aggregation of the vesicles. The preparation was then placed in a discontinuous sucrose gradient in which the first layer, at a density of 1.049 g/ml, consisted of buffered sucrose, KCl, and pyrophosphate over a cushion of higher density buffered sucrose. Following centrifugation at 30,000*g* for 30 min at 4°C, a band of enriched *T. cruzi* PMV formed at the interface. Similar results were obtained with *T. cruzi* metacyclic trypomastigotes. The tritium-labeled glycoconjugates were enriched approximately 3.3–4.0 times by this procedure (PMV/TH ratios). Storage of the *T. cruzi* PMV preparation was at a 10% suspension (v/v) in 0.29 *M* sucrose, 0.02 *M* histidine (pH 7.4) at −70°C. With these storage conditions there was no significant loss of magnesium-ATPase or of adenylyl cyclase activity for at least 3 months.

Electron Microscopy. Transmission electron microscopy of the cross-sections of the thickness of the pellets of *T. cruzi* PMV (n=3) revealed a packed population of empty vesicles of different sizes without a layering effect from top to bottom (Fig. 1A). Importantly, no microtubules or flagellar structures were identified inside or outside the vesicles. High magnification study showed there was a predominance of larger vesicles which frequently showed focal loss of the unit membrane which was replaced by amorphous osmiophilic proteinaceous material (Fig. 1, B and C).

Sidedness Assay. Sidedness of *T. cruzi* PMVs was determined by solid-phase immunoassay and by solid-phase sialic acid chromophore release assay. Utilizing metabolically labeled epimastigotes with [^{35}S]

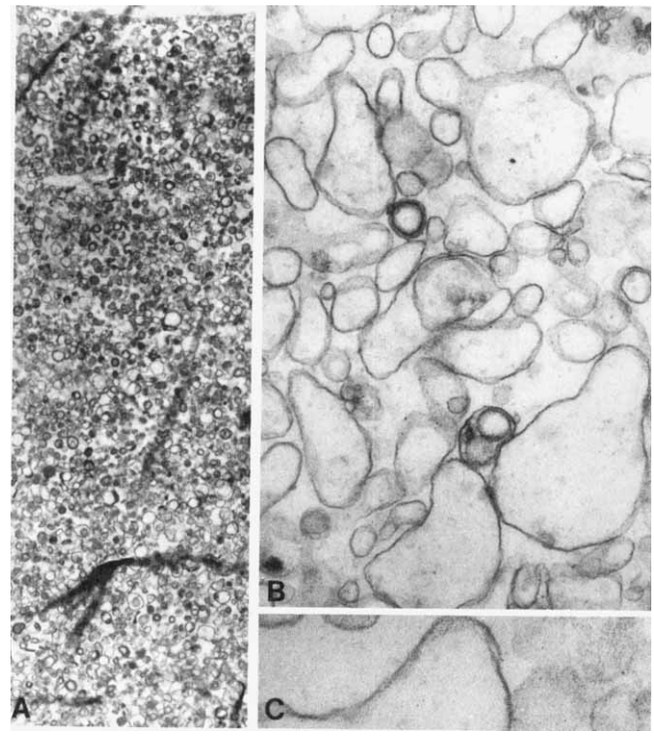

Figure 1. (A) Ultrastructure of *T. cruzi* PMV. This is a low-power electron micrograph of the entire thickness of one representative P_6 pellet obtained after 100,000*g* for 60 min. The pellet was subsequently fixed in glutaraldehyde for electron microscopy. Note that the vesicles are uniformly distributed from top to bottom (original magnification ×13,340). (B) and (C). Heterogeneous-sized and shaped *T. cruzi* plasma membrane vesicles. Note predominant larger vesicles and smaller, more uniform vesicles. Not all vesicles are totally enclosed by well-defined unit membranes and the outer membrane of many is focally attenuated and replaced by amorphous osmiophilic protein (original magnification B, ×66,700; C, original magnification ×133,400).

methionine, solid-phase rabbit IgG anti-*T. cruzi* SAg bound 80.1% ± 2.7% of the PMV preparation (mean ± SEM, $n = 3$). When the solid-phase antibody to the epitopes on both sides of the membranes was used, 92.1% ± 4.2% of the radioactivity was precipitated (mean ± SEM, $n = 3$) and these data indicate that the estimate of sidedness was to within 8%. Sidedness and purity were confirmed when a similar result was obtained by measuring the sialic acid chromophore released after treatment with solid-phase neuraminidase in the presence and absence of Triton X-100 (RSO: 86.6% ± 5.7%, mean ± SEM, $n = 6$).

Permeability to [^{14}C]Dextran. The determination of the ^3H_2O space in three different *T. cruzi* PMV preparations yielded 2.98 ± 0.04 μl/mg (mean ± SEM, $n = 3$). The [^{14}C]dextran space in the same preparations was 3.26 ± 0.07 μl/mg (mean ± SEM, $n = 3$). The ratio of the dextran space over the water space was 1.09.

Extraction of Nucleotides. *T. cruzi* PMV preparations were not significantly contaminated with nuclear or kinetoplast DNA, nor were they contaminated with RNA. When *T. cruzi* flagellates were metabolically labeled with [^{3}H]thymidine and [^{3}H]uridine, DNA and RNA were virtually completely extracted.

Enzyme Markers. The addition of 50 ng of *T. cruzi* PMV/well of confluent *L6* myoblasts resulted in no specific release of the surface protein ^{51}Cr label for up to 6 hr of incubation in physiologic conditions with oxygenated KRB. Similar negative release assay results were obtained when the surface glycoproteins of *L6* myoblasts were metabolically labeled with [^{3}H]glucosamine. The ability of *T. cruzi* PMV to hydrolyze ATP in the absence of magnesium or calcium ions was very low. When magnesium was added, a powerful ATPase was activated which was not stimulated by increasing concentrations of potassium ions (Table I). The magnesium-ATPase was also not significantly stimulated by the addition of sodium and potassium ions (100:5 ratio) (11.5% increase of total magnesium-ATPase) and this activity was not appreciably inhibited by 0.1 m*M* ouabain (12.4% of total magnesium-ATPase). The magnesium-ATPase activity was oligomycin resistant in the concentrations tested (86.4% of total magnesium-ATPase) and calcium ions did not significantly substitute for magnesium with these membrane preparations (15.8% of total magnesium-ATPase; 1 m*M* Mg^{2+} restituted 90.7% of activity). Adenylyl cyclase activity was detected in *T. cruzi* total homogenates and this activity was unmasked in the homogenate preparations by 1.0 m*M* NaF and Lubrol PX (2.5 μg/μg PMV). The adenylyl cyclase of the extracted *T. cruzi* PMV, however, appeared unmasked because NaF and Lubrol PX were not capable of stimulating it further (Table II). In sharp contrast acid phosphatase assays indicated that this enzyme activity had been extracted from the PMV preparations and PMV to TH ratios were 0.003 ± 0.0004 (mean ± SEM, $n = 3$).

Permeability to ^{86}Rb. Intact, freely swimming epimastigotes concentrated ^{86}Rb when magnesium-ATP was added to the upper chamber and this phenomenon was stopped on addition of EDTA. *T. cruzi* PMV preparations, however, did not have the capacity to concentrate ^{86}Rb in similar conditions of assay (Fig. 2).

Nature of the Contaminating Membrane Fraction. The immunoassay used indicated that 18–20% of the *T. cruzi* PMV was a contaminating fraction. *T.*

Table I. ATPase Activity of *T. cruzi* PMV (n*M* Pi $min^{-1}mg^{-1}$)[a]

Mg^{2+} ATPase	+1 m*M* KCl	+5 m*M* KCl	+10 m*M* KCl	+100 m*M* KCl
322.6 ± 11.4	357.2 ± 22.1	332.9 ± 14.9	326.1 ± 19.1	334.3 ± 2.5
Oligomycin sensitive Mg^+ + ATPase	(Na^+/K^+) stimulated Mg^{2+} ATPase		Ouabain sensitive (Na^+/K^+) Mg^{2+} ATPase	
43.9 ± 8.7	37.0 ± 10.3		40.1 ± 17.4	
Ca^{2+} ATPase	+1 m*M* Mg^+			
51.1 ± 6.2	292.8 ± 8.6			

[a] The experiments were carried out as described with the following additions: Mg^{2+}, as $MgCl_2$, 1 m*M*; Ca^{2+}, as $CaCl_2$, 1 m*M*; Na^+, as NaCl, 100 m*M*; K^+, as KCl, 5 m*M*; oligomycin and ouabain, 0.1 m*M*. Mean ± SEM, $n = 6$ separate experiments, each determination in triplicate.

Table II. Latent Enzymatic Activity (n*M* cAMP min^{-1} mg^{-1})

	TH	P_4	PMVs	PMV/TH
Adenylyl Cyclase[a]				
No treatment	1.95	3.81	3.95	2.03
+1 m*M* NaF	3.88	3.92	3.83	
+2.5 μg of Lubrol PX μg^{-1}	4.0	3.85	4.00	
Acid *p*-nitrophenyl phosphatase[b]				
0.1% w/v Triton X-100	11.8	0.032	0.038	0.0032

[a] Forty micrograms of protein, 37°C × 15 min, mean ± 0.89% SEM, $n = 3$.
[b] One-hundred micrograms of protein, 37°C × 60 min, mean ± 9.1% SEM, $n = 3$.

cruzi PMV contained NADH oxidase with a putative electron acceptor and this baseline activity was stimulated further by ubiquinone-0, cytochrome *c*, and the nonbiologic electron acceptor $K_3Fe(CN)_6$. *T. cruzi* PMV also contained cytochrome *c* oxidase activity (Table III).

Activation of the Alternate Pathway of Human Complement. Activation of the alternative pathway of

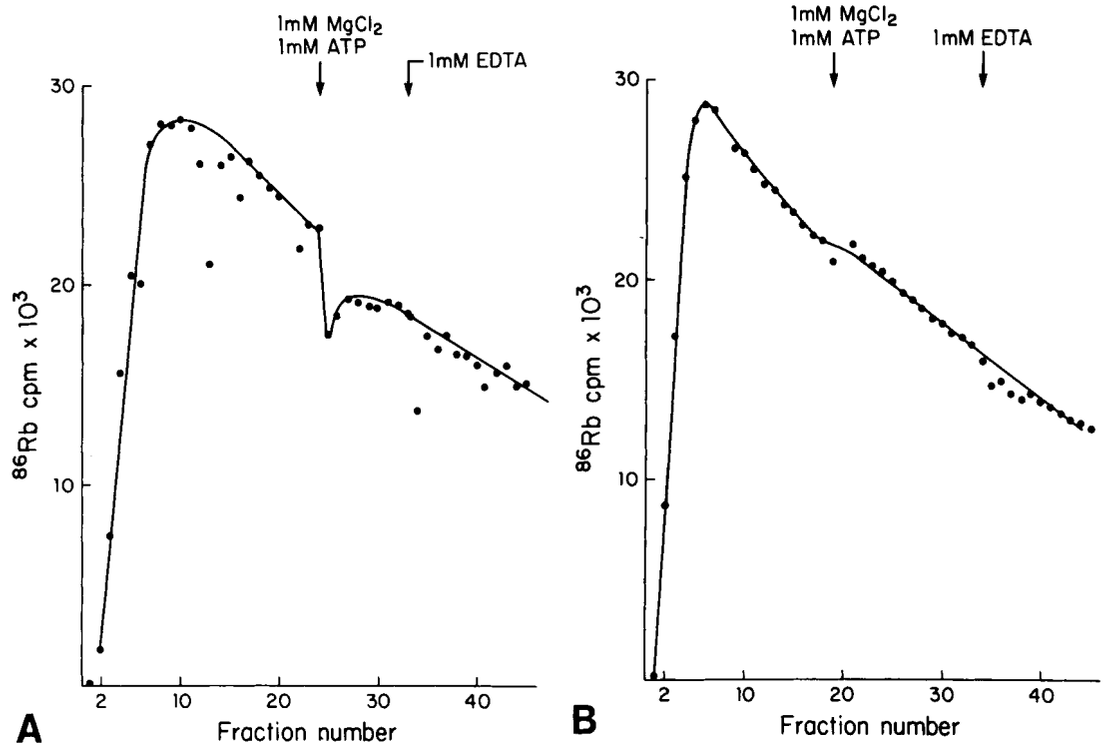

Figure 2. (A) Magnesium-ATP-dependent uptake of ^{86}Rb by flow dialysis. The assay shown was done at pH 7.4 at 28°C in a basal isosmolar 0.295 *M* sucrose, 5 m*M* glucose, and 10 m*M* histidine buffer. Epimastigotes (10^8), previously washed and equilibrated in the basal buffer, were placed in the upper chamber that was separated from the lower by a membrane. Five microliters of ^{86}Rb (1 mCi/ml; Amersham) at 8.6×10^7 cpm/ml were added to the upper chamber and equilibration was quickly measured in the pumped effluate from the lower chamber across a Spectrapor 1 dialysis membrane (*M*, 600–800 cutoff). The basal buffer was passed in the lower chamber at 6 ml/min by a Pharmacia P3 pump. As indicated by the arrows, 1 m*M* magnesium-ATP (final concentration) resulted in ^{86}Rb uptake which was quickly abrogated upon the addition of 1 m*M* EDTA (final concentration). (B) Magnesium-ATP-dependent uptake of ^{86}Rb by flow dialysis. Failure of ^{86}Rb uptake by *T. cruzi* PMV upon addition of magnesium-ATP. When the experiment described in 5A was repeated using 500 μg of *T. cruzi* P_6 pellet membranes, no magnesium-ATP-dependent ^{86}Rb uptake was demonstrated.

Table III. NADH Oxidase Activity in *T. cruzi* PMV

NADH (mol)	Electron acceptor	PMV specific activity (V_0) (μM NAD min^{-1} mg^{-1})
1.7×10^{-4}	None	1.29×10^{-5}
	Ubiquinone 0 (6.0×10^{-5} *M*)	1.12×10^{-4}
	Cytochrome *c* (3.0×10^{-6} *M*)	1.11×10^{-4}
	$K_3Fe(CN)_6$ (4.6×10^{-5} *M*)	86.6×10^{-3}

Mean ± 0.18–10.4% SEM, $n = 3$

Cytochrome *c* oxidase activity in *T. cruzi* PMV

Cytochrome *c* (mol)	Specific activity (V_0) (μM min^{-1} mg^{-1})		
	TH	P_4	PMV
0.015	0.295	4.87	5.91

Mean ± 6.1% SEM, $n = 3$

complement was demonstrated by the appearance of the Bb activation fragment of Factor B in whole human serum incubated with *T. cruzi* PMV prepared from epimastigotes and inulin, the known activator (B and E of Fig. 3) but not in serum incubated without activator (sterile saline) (F). Factor B-related antigens capable of cross-reacting with the employed anti-Factor B antiserum were not present in the vesicle preparation (D).

Discussion

The results of the experiments reported here describe a procedure for the production of PMV derived from flagellates of *T. cruzi*. The method may be applicable to other protozoa. The method resulted in a high yield (1 mg/4 × 10^{10} flagellates). Up to 82% of the preparation was shown to be derived from the plasma membrane and most, if not all, of these vesicles were right-side out by immunoassay with immobilized IgG α *T. cruzi* surface antigen and by solid-phase neuraminidase sialic acid chromophore assays after solubilization with detergent. The *T. cruzi* PMV preparations showed a population of heterogeneous-sized vesicles by transmission electron microscopy and the limiting membrane of these vesicles was frequently focally attenuated and replaced by amorphous osmiophilic proteinaceous material. Study of the $^{3}H_2O$ and [^{14}C]dextran spaces showed that *T. cruzi* PMV were not sealed to dextran M_r 70,000. These data were supported further by the observation that *T. cruzi* PMV preparations were not capable of concentrating ^{86}Rb in flow dialysis assays. These results indicate that *T. cruzi* PMV preparations are not suited for ion transport studies. Approximately 18–20% of the *T. cruzi* PMV preparation described here was a contaminating membrane fraction, marked by NADH oxidase activity and by cytochrome *c* oxidase activity. About 13.6% of the magnesium-ATPase was oligomycin sensitive and this value may correspond to the degree of kinetoplast membrane contamination. We believe that these enzyme markers showed that the principal contaminants were from kinetoplast and endoplasmic reticulum membranes. Other investigators have devised methods for the production of plasma membranes of *T. cruzi* (6, 22, 23); however, these procedures have had the disadvantages of either low yields after severe physical and chemical treatment and/or contamination by flagellar and tubular structures and by acid hydrolases.

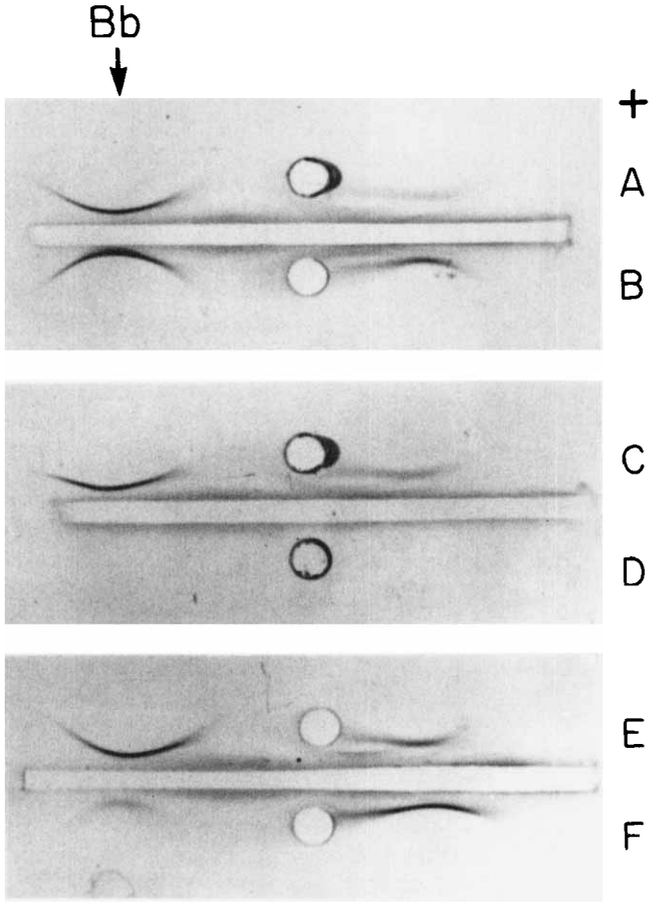

Figure 3. Activation of the initial step of the alternative pathway of human complement. Immunoelectrophoreses of *T. cruzi* epimastigote PMV-treated fresh human serum resulted in the specific separation of the Bb fragment from Factor B (arrow).

The right-side-out *T. cruzi* PMV preparation described here was enriched with a characteristic oligomycin resistant ouabain-insensitive magnesium-ATPase which is an apparent marker of *T. cruzi* plasma membranes (24). The specific activity of the *T. cruzi* PMV magnesium-ATPase was somewhat higher than values obtained in other preparations by other investigators (6, 22, 23). The *T. cruzi* homogenate also contained an adenylyl cyclase which was stimulated when treated with the nonionic detergent Lubrol PX or sodium fluoride. Adenylyl cyclase is considered an enzyme marker for mammalian plasma membranes. However, *T. cruzi* PMV showed an unmasked adenylyl cyclase which was not stimulated further by Lubrol PX or by NaF, probably because the membranes were rendered permeable to the substrate by the extraction procedure. Other investigators have shown *T. cruzi* membrane preparations with a higher adenylyl cyclase activity but those preparations were also enriched with a contaminating acid phosphatase (7, 22, 23). Our lower adenylyl cyclase activity probably reflects extraction of some of the enzyme in the course of the preparation of a more enriched plasma membrane fraction.

One might consider that host cell penetration during parasitosis may be facilitated by plasma membrane bound glycoproteolytic enzymes. In this regard, a soluble *T. cruzi* neuraminidase capable of removing sialic acid from the surface of monolayers of myocardial and endothelial cells may be implicated in parasitosis (25). Contrary to this consideration, however, *T. cruzi* PMV preparations did not show the capacity to remove surface glycoconjugates of host myoblast monolayers when

incubated under oxygenated physiologic conditions as measured by sensitive ^{51}Cr and [^{3}H]glucosamine release assays. It is possible, however, that these putative membrane bound enzymes may have been extracted and the experimental design reported here does not completely rule out this possibility.

The *T. cruzi* PMV preparation described here was biologically active. In a recently published study, we showed, after extensive investigation, that these membranes specifically adhered to *L6* myoblast host cells preferentially to smooth muscle and epithelial cells as a function of time, surface area, and concentration in saturation phenomena related to the existence of complementary parasite attachment molecules with the capacity to bind host cell surface receptors (3). In this study, we have shown, in addition, that *T. cruzi* PMV activated the alternate pathway of human complement in a manner similar to that described for living flagellates (26, 27). Recent investigations suggest that complement activation may be implicated in the ability of *Leishmania major* to parasitize host macrophages (28). Whether complement activation plays a role in the *T. cruzi* penetration of host striated muscle is not known and this is an area for further investigation.

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