In Situ Compositional Analysis of Acidocalcisomes in Trypanosoma cruzi*

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We measured the elemental content of different compartments in Trypanosoma cruzi epimastigotes using quick freezing, ultracryomicrotomy, and electron probe microanalysis. Vacuoles identified by high electron density contained (in units of mmol/kg dry weight ± S.E.) large amounts of phosphorus (1390 ± 13), magnesium (646 ± 19), calcium (171 ± 5), sodium (161 ± 18), and zinc (148 ± 6). No other compartment had appreciable calcium or zinc content. Iron (128 ± 16 mmol/kg) was detected only in vacuoles distinct from the electron-dense vacuoles and other organelles. Incubation of cells for 70 min in culture medium in the presence of ionomycin plus nigericin led to a very significant 3- or 2-fold increase in potassium in the electron-dense vacuoles and the iron-rich vacuoles, respectively, with no significant change in the other elements investigated. This indicated the acidic nature of the vacuoles and demonstrated that the electron-dense vacuoles corresponded to what were described previously as acidocalcisomes, i.e. acidic compartments rich in Ca\(^{2+}\). The acidocalcisomes were investigated by separation of epimastigote fractions on Percoll gradients in combination with Triton WR-1339 treatment. This detergent caused a rapid vacuolation; these vacuoles were shown by electron microscopy to be largely transparent, with a diffuse matrix. Percoll gradient fractionation demonstrated decreases in the density of various organelle markers in detergent-treated cells compared with controls. Large decreases in the density of the acidocalcisome and the mitochondrion were seen, as well as smaller decreases in the density of the other markers. Conventional electron microscopy of epimastigotes loaded with gold-labeled transferrin indicated that the endosomal system was separate from vacuoles that probably corresponded to the calcium-containing organelles detected by electron probe microanalysis. The combined results provide evidence that acidocalcisomes are organelles different from lysosomes or other organelles previously described in these parasites.

Invasion of host cells by Trypanosoma cruzi, the etiologic agent of Chagas’ disease, is dependent upon an elevation in the concentration of cytosolic free calcium in the invading trypanostigote (1). Unlike mammalian cells, T. cruzi possesses most of its intracellular Ca\(^{2+}\) in an acidic compartment named the acidocalcisome (2). The biochemical characterization of this organelle has provided evidence that it is acidified by a vacuolar-type proton-translocating (V-H\(^{+}\))-ATPase and that it has a Ca\(^{2+}/H^{+}\) countertransporting ATPase for Ca\(^{2+}\) uptake (2). Acidocalcisomes have also been found in other trypanosomatids such as Trypanosoma brucei (3-5) and Leishmania mexicana amazonensis (6) and in Toxoplasma gondii (7). This organelle is in various aspects similar to the vacuole present in fungi and plant cells (8) but apparently has no counterpart in animal cells.

Using fluorescence microscopy it was possible to detect bafilomycin A\(_{1}\)-, nigericin-, and NH\(_{4}\)Cl-sensitive acridine orange accumulation in round vacuoles of varying size located mainly to the posterior and anterior of T. cruzi epimastigotes (2) and T. brucei procyclic trypanostigotes (3), and these vacuoles were suggested to correspond to the acidocalcisomes (2, 3).

Several authors (9–11) have reported the presence of calcium-rich inclusion vacuoles in different trypanosomatids following energy-dispersive x-ray microanalysis. Vacuoles observed in Trypanosoma cyclops were tentatively termed polyphosphate bodies on the basis of a high phosphorus content, an appearance similar to algal polyphosphate bodies and the prior report of polyphosphates in other trypanosomatids. They had an appreciable calcium content and, possibly, some zinc, although their composition was not quantified (9). T. cruzi epimastigotes were shown to possess electron-dense organelles identified by scanning transmission electron microscopy of whole cells. These contained large amounts of magnesium, potassium, calcium, phosphorus, and zinc (10). Iron was also found in these studies although its co-localization with the other elements detected was not so evident. Electron probe x-ray microanalysis of Leishmania major promastigotes demonstrated that their electron-dense vacuoles are also rich in phosphorus, magnesium, calcium, sodium, and zinc, with concentrations of chloride and potassium below that of the cytoplasm (11). Incubation of promastigotes in the presence or absence of glucose did not cause significant changes in the vacuolar content of phosphorus, calcium, magnesium, or zinc, but changes in potassium and chloride were observed in both electron-dense vacuoles and in the cytoplasm (11).

In this work we used quick freezing, ultracryomicrotomy, and electron probe x-ray microanalysis (12–15) to study the elemental composition of electron-dense vacuoles in T. cruzi, with or without prior treatment with ionophores. Our results suggest that the electron-dense organelles previously identified

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in T. cruzi epimastigotes (10) correspond to acidocalcisomes (2). In addition, subcellular fractionation and gold-labeled transmembrane studies of T. cruzi epimastigotes provide evidence that these organelles are different from lysosomes or the prelysosome vacuoles previously described as reservosomes (16).

**Experimental Procedures**

**Culture Methods—**T. cruzi clone Silvio X10/4 (17) epimastigotes were maintained at 26°C in LIT medium (18) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml hemin and harvested for electron probe x-ray microanalysis in one exponential growth phase. T. cruzi Y strain (19) epimastigotes were grown at 26°C in a liquid medium consisting of brain-heart infusion (37 g/liter), 100 units/ml penicillin, 100 μg/ml streptomycin, hemin chloride (20 mg/liter dissolved in 50% triethanolamine), and 10% heat-inactivated newborn calf serum (21). After five days in inoculation, T. cruzi strain cells were collected by centrifugation, washed twice with Dubelco’s phosphate-buffered saline (138 mM NaCl, 2.7 mM KClPO₄, 8.1 mM Na₂HPO₄, pH 7.2), and were used for the fractionation studies.

**Chemicals—**Arsenazo III, fetal and newborn calf serum, nigericin, EGTA, sodium dithionite, leupeptin, RNase, DNase, glucose-6-phosphate dehydrogenase (type XXIII), succinate, benzoyl-Pro-Phe-Arg-p-nitroanilide, p-nitrophenyl phosphatase, gold chloride, polyethylene glycol, and Trionit WR-1339 were purchased from Sigma, 5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester and transferrin (bovine “holoenzyme”) were bought from Boehringer Mannheim; nigericin was from Calbiochem; Percoll was from Pharmacia Biotech Inc., and silicon carbide was from Aldrich. Other reagents were analytical grade.

**Elemental Microanalysis—**Epimastigotes were harvested and resuspended at 2 × 10⁷ cells/ml in fresh growth medium (LIT) to maintain their morphology. Portions of the suspension were incubated in the absence (control) or presence of 2 μM ionomycin plus 4 μg/ml nigericin for 70 min at room temperature (22°C). After incubation the cells were concentrated by centrifugation, washed once in LIT plus 0.5% fetal calf serum, and centrifuged for 1 min at 14,000 × g in an Eppendorf centrifuge to compact the pellet. The supernatant liquid was removed, and the cells were transferred by Pipetman to the end of wooden sticks for freezing in liquid ethane. The cells were quick frozen by plunging them into liquid nitrogen-cooled liquid ethane at approximately −185°C in a Leica KF80 plunge freezing device (Leica, Vienna, Austria); frozen blocks were subsequently stored under liquid nitrogen.

**Cryosectioning** was performed on a Lecia UltraCt microscope equipped with an FC-4E cryosectioning attachment and an ionization antistatic device (Hauf Static Line, Diatome). Blocks were first trimmed to ~200 × 300 μm using a diamond trimming tool, and then frozen thin sections were cut at a specimen temperature of ~160 to ~165°C using a 35° diamond knife kept at a temperature of ~155°C. Sections from each ribbon cut by the microtome were collected by means of an eyelash probe and placed into a hexagonal-carbon-coated grid. This was covered with a second coated grid, sandwiched between the leaves of a small indium foil envelope, and pressed to attach the grid. This was covered with a second coated grid, sandwiched between the leaves of a small indium foil envelope, and pressed to attach the sections to the support film (20). Specimens were stored in grid boxes under liquid nitrogen. Sections were analyzed in a VG Microscopes HB501 scanning transmission electron microscope (VG Scientific, Beverly, MA) as described previously (21). Grid pairs were removed from storage and were separated under liquid nitrogen. A single frozen hydrated grid was cryotransferred, freeze-dried at about ~110°C, and subsequently re-cooled to approximately ~160°C for analysis. Dark field images were recorded digitally at low electron dose (<10⁶ e/nm²) by means of a Gatan Digital scrap (Gatan Inc., Pleasanton, CA) interfaced to an Apple Macintosh Quadra 950 computer. Images were analyzed with the Gatan Digital Micrograph program. Energy-dispersive x-ray spectra were acquired with a Tracor Northern TN 5500 multichannel analyzer and Micro-ZHV ultrathin window detector (Noran, Middleton, WI) having a collection solid angle of 0.18 sterad. The spectra were transferred to a Quadra 950 computer and were processed using the program Desktop Spectrum Analyzer (22). A Simplex non-linear least squares fitting routine and the Hall peak/continuum method were used to quantify the elemental composition in terms of millimoles per kg dry weight (14). Standards were prepared by analyzing dried cryosections of rapidly frozen solutions containing known concentrations of salts and polyvinylpyrrolidone as an organic matrix. Iron and zinc were quantified by calculating sensitivity factors relative to potassium with the Desktop Spectrum Analyzer program. Electron energy loss spectra (EELS) were recorded with a Gatan model 666 parallel electron spectrometer (23) and the Gatan ELP acquisition program running on a Quadra 950 computer (24). EELS spectra were acquired with an integration time of 0.3 s and 50 read-outs of the photodiode array; the electron dose was approximately 10⁸ e/nm². Estimated ratios of nitrogen, oxygen, phosphorus, and calcium in the calcium-rich vacuoles were obtained by means of the EELS quantification and analysis program in the ELP software. Spectra were collected from granules with mass thickness below one inelastic mean free path at 100 keV beam energy to avoid background subtraction errors due to plural inelastic scattering (25, 26).

**Cell Loading with Triton WR-1339 and Surface Labeling—**Epimastigotes collected from culture by centrifugation were resuspended at about 3 × concentration (approximately 10⁸ cells/ml in Dubelco’s phosphate-buffered saline, pH 7.2, containing 10 mg/ml Triton WR-1339 and incubated at 30°C for 50 min. This resulted in, on average, 84% vacuolation in treated cells versus less than 5% in untreated cells, as observed by light microscopy (n = 8). Cells were recovered by centrifugation, resuspended in 3 ml of 20 mM Hepes, 5 mM KCl, 125 mM NaCl, pH 8.0, with 0.53 mM 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester and centrifuged at 160 to 200 × g for 5 min. The supernatant fraction was centrifuged for 40 min at 105,000 × g. The pellet was recovered in resuspension buffer (lysis buffer without DNase, RNase, or leupeptin) with passage several times through 23-gauge needle. This fraction was applied to an 18-ml density gradient containing 34% Percoll, 34% 0.5 mM sucrose, and 32% resuspension buffer and centrifuged for 50 min at 69,500 × g (max) in a Beckman 70Ti rotor. Gradients were fractionated by upward displacement with 2.25 mM sucrose.

**Assay for Acidic Calcium Storage—**Calcium accumulation in the presence of ATP was assayed by an end point assay based on previous methods (2–4). Fractions were incubated in a mixture of 10 mM Hepes, 65 mM KCl, 125 mM sucrose, 2 mM MgCl₂, 50 mM EGTA, 90 μM EDTA, 80 μM CaCl₂ (free Ca²⁺ 4 μM; Ref. 2), 1 mM ATP, 2 μg/ml oligomycin, 1 μg/ml antimycin A, 1 μM ionomycin, 40 μM arszeno III, pH 7.2. The oligomycin and antimycin A prevented uptake of calcium into mitochondria, and the ionomycin released calcium from other non-acidic compartments (2–4). After 15 min incubation at 30°C in a water bath, mixtures were transferred into an SME-Amino DW2000 dual wave-length spectrophotometer, and the calcium accumulated in acidic compartments was released by the addition of 5 μM nigericin (measured with arsenazo III 675–685 nm; Ref. 2).

**Subcellular Compartment Marker Assays—**The following marker assays were used: for lysosomes, acid phosphatase, α-mannosidase, and cysteine proteinase; for mitochondria, NADH-succinate-cytochrome c reductase; for glycosomes, hexokinase; for cytosol, glucose-6-phosphate dehydrogenase; for cell surface, measurement of surface fluorescent labeling. Acid phosphatase, α-mannosidase, and acid protease activities were assayed by modifications of previous methods using 96-well microtiter plates (28). For acid phosphatase, the sample (20 μl) was incubated for different periods at 30°C with 50 μl of 10 mM p-nitrophenyl phosphate in 0.1 M sodium acetate, pH 5.5, before the reaction was stopped by the addition of 100 μl of 150 mM sodium hydroxide. α-Mannosidase was assayed similarly with 5 mM p-nitrophenyl α-D-mannopyranoside in 0.1 M potassium Mes, pH 5.5, with 60 mM sodium hydroxide as the stop solution. Samples for protease assay were mixed with 10 mM sodium Mes, pH 5.5, 1 mM thiol protease, and the supernatant fraction used in the assay, with the addition of 5 mM dithiothreitol and 0.1 mM benzoyl-Pro-Phe-Arg-p-nitroanilide. The plate was incubated at 30°C and read intermittently. Under these conditions,

1 The abbreviations used are: EELS, electron energy loss spectra; Mes, 4-morpholinethesulonic acid.
assay conditions, it was confirmed that the protease activity was sensitive to a 40 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane (E64; results not shown) and therefore corresponded to the lysosomal cysteine proteinase cruzipain (29, 30) and not the cytosolic alkaline protease that is insensitive to this inhibitor (31). The release of p-nitrophenol or p-nitroaniline from substrates was measured using a Dynatech MR5000 reader at 410–450 nm. Possible surface localization of these enzymes was tested by assaying intact cells in reaction mixtures as above, followed by removal of cells, re-incubation of the assay mixture to determine released activity, and lysis of cells (by freeze-thaw in dry ice/ethanol) and assay of lysates to determine total activity. The cell surface was labeled with a fluorescein reagent as described above, and fractions were assayed by 20-fold dilution in water followed by measurement of fluorescence (λ ex 490 nm; λ em 520–460 nm) in a Hitachi F-2000 fluorimeter. NADH-succinate-cytochrome c reductase activity was determined by the method used in Ref. 32, using succinate as the substrate and following the reaction at 550–540 nm in the dual wavelength spectrophotometer. This enzyme has previously been detected in T. cruzi epimastigote mitochondrial fractions, using NADH as substrate (33). Hexokinase (34) was assayed at 30 °C in a mixture of 10 mM glucose, 0.6 mM ATP, and added glucose-6-phosphate dehydrogenase, and with the omission of glucose, and cytoplasm (C). Note that a single membrane surrounds some of the electron-dense vacuoles (arrows). Insets show 2 × magnifications of a calcium-rich vacuole showing a limiting membrane (upper left) and an iron-containing vacuole (lower left). Bar = 1 μm.

**FIG. 1.** Transmission electron micrograph of epimastigotes. Unstained cells were prepared by quick freezing and ultracytomicotomy as described under "Experimental Procedures." The cellular compartments that were analyzed by energy-dispersive x-ray microanalysis included the nucleus (N), mitochondria (M), kinetoplast-DNA (K), electron-dense vacuoles (arrows), iron-rich vacuoles (Fe), and cytoplasm (C). Note that a single membrane surrounds some of the electron-dense vacuoles (arrowheads). Insets show 2 × magnifications of a calcium-rich vacuole showing a limiting membrane (upper left) and an iron-containing vacuole (lower left). Bar = 1 μm.

**Labeling of Epimastigotes with Gold-Transferrin—Colloidal gold (15 nm diameter) was prepared by the method of Frens (35). Gold suspension (0.1 mg/ml, pH 6.0) was added to transferrin to give a final transferrin concentration of 25 μg/ml (optimal concentration determined as per Ref. 36). Colloidal gold-transferrin was recovered and washed by centrifugation (64,000 × g max; 30 min) in 0.6 mg/ml polyethylene glycol compound, 20 mM Mes, pH 6.0. Cells were washed 3 times in Dulbecco’s phosphate-buffered saline, resuspended in 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 50 mM HEPES, 5.5 mM glucose, pH 7.2 at 3 × 107 cells/ml, and loaded with the gold complex for 1–8 h at 30 °C, with the amount of gold added being equivalent to a final A520 of 0.2–0.3, before fixation for electron microscopy.

**Conventional Electron Microscopy—**Control, Triton WR-1339-treated, and gold-transferrin-loaded epimastigotes were fixed in 2.5% glutaraldehyde, 0.1 M K+-HEPES buffer, pH 7.5, for 40 min at room temperature and resuspended in 0.1 M K+-HEPES buffer, pH 7.5, 50 mM sucrose, before post-fixation in 2% OsO4 in water using a microwave oven (Ted Pella 3440): two treatments at 42 °C (max), each 8 s on, 20 s off, and 8 s on, with a 15-min incubation at room temperature after each treatment. Specimens were then stained en bloc with 3% potassium ferricyanide for 10 min, rinsed in water, and incubated in saturated uranyl acetate for 30 min (sample in uranyl acetate was microwaved at the beginning of the incubation), dehydrated in ethanol/propanol ox-ide, and embedded in Lx112 Epon substitute. Ultrathin sections were made in a Reichert Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a JEOL 100CX electron microscope operating at 80 kV. For imaging whole epimastigotes, these were suspended in Dulbecco’s phosphate-buffered saline, pH 7.4. Drops were applied to Formvar-coated grids, and cells were allowed to adhere for 10 min and then were carefully blotted dry and observed directly with the JEOL electron microscope.

**Statistical Analysis—**Elemental concentrations are expressed as

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means ± S.E. for n measurements. Statistical significance between different treatments was determined by Student's t test. Significance was considered at the p < 0.001 level for potassium changes in the calcium-rich vacuoles and at the p < 0.01 level for potassium changes in the iron-rich vacuoles.

RESULTS

Elemental Analysis of T. cruzi Epimastigotes—Scanning transmission electron micrographs of rapidly frozen, cryosectioned, and freeze-dried epimastigotes showed very good ultrastructural preservation (Fig. 1). Easily identified structures include the mitochondrion with the kinetoplast-DNA, subpellicular microtubules, flagellar microtubules, the nucleus, and (not shown in this figure) endoplasmic reticulum and the Golgi. The contrast of a given structure in the image arises solely from its mass density since these preparations were not stained. In addition, there were various organelles unidentifiable by their appearance, in comparison with conventional (chemically fixed) electron microscopy preparations (see below). The most prominent of these were spherical vacuoles of high mass density, as reflected by their marked electron opacity compared with other compartments (Fig. 1, arrowheads). These electron-dense vacuoles have an average diameter of 200 ± 90 nm (± S.D.). A single membrane surrounding the electron-dense material was clearly seen in some of these vacuoles (Fig. 1, arrowheads, and upper left inset). In each longitudinal cryosection 1–4 of these vacuoles were found per cell. Since the average cross-section of an epimastigote is about 3–4 μm, and the section thickness was approximately 100 nm, there are 30–40 longitudinal sections per cell. Therefore a 200-nm vacuole would be visible in 2–3 sections; the range in vacuole number per cell is 10–60, with an average of 30. Similar electron-dense vacuoles were seen when whole epimastigotes were observed by transmission electron microscopy without fixation (Fig. 2). More than 40 electron-dense vacuoles of varying sizes are observed in each of the epimastigotes shown in Fig. 2. X-ray spectra were acquired from nuclei, mitochondria, kinetoplast-DNA (dense region), electron-dense vacuoles, iron-rich vacuoles, and cytoplasmic spaces of cryosections. Typical x-ray spectra from these compartments are shown in Fig. 3, a–f, for the control and Fig. 3, g and h, for the nigericin/ionomycin-treated sample (see below). Quantification of spectra from nuclei (Fig. 3a), cytoplasm (Fig. 3b), and mitochondria (Fig. 3c) gave similar ion concentrations as in many other cell types (e.g. Refs. 12–15). Quantitative analysis of the electron-dense vacuoles indicated that their mass density was due to very high amounts of phosphorus, magnesium, calcium, sodium, and zinc (Fig. 3, c and g). The electron-dense vacuoles were the only compartment with marked calcium and zinc content, whereas iron was only detected in larger vacuoles that were less electron-dense (Fig. 1, lower left inset, and Fig. 3, f and h). It is important to mention that x-ray microanalysis measures total calcium rather than free calcium.

The elemental content of the various compartments examined in the control cells was determined in units of millimole/kg dry weight, and the results of one experiment are shown in Table I, although analysis was performed on samples from two independent experiments. Results were comparable except that the sodium content in most organelles, and the calcium content of the calcium-rich vacuoles, was about 2-fold higher in one experiment (data not shown). Generally, in the electron-dense vacuoles, potassium and chloride levels were lower than in the cytoplasm. The low sulfur level in these vacuoles indicates that they do not contain much cysteine or methionine or proteins containing these amino acids. Analysis of EELS (Fig. 4) from 11 electron-dense vacuoles in nigericin/ionomycin-treated cells (see below) yielded a phosphorus-to-oxygen ratio of 0.11:1 (±15% S.E.) and a nitrogen-to-oxygen ratio of 0.065:1 (±13% S.E.). Because spectra were acquired at a relatively high dose of 10^5 e/nm², it is likely that significant oxygen was lost due to beam damage, so these measured P:O and N:O ratios represent upper limits. As discussed later, the results suggest high levels of carbohydrate in the vacuolar matrix.

Changes in the Elemental Composition of Epimastigotes after Incubation in the Presence of Ionomycin and Nigericin—Previous studies (2) demonstrated that acidic calcium storage organelles in T. cruzi (acidocalcisomes) are the most important Ca²⁺-containing compartments in these parasites. In the presence of EGTA to prevent refilling of internal stores, Ca²⁺ can be released from acidocalcisomes by combined treatment with a calcium ionophore, such as ionomycin, and a K⁺/H⁺ exchanger such as nigericin. This is because nigericin binds essentially no calcium below pH 7.0 and cannot transport Ca²⁺ out of acidic compartments because of competition from protons at the inside face of the membrane (37). In the absence of nigericin, ionomycin releases a relatively small amount of Ca²⁺ only from neutral or alkaline compartments in T. cruzi but releases much more Ca²⁺ after nigericin has elevated the pH of acidic compartments. To determine whether treatment with ionomycin plus nigericin could alter the elemental composition of the electron-dense vacuoles and demonstrate that they correspond to what has been described before as acidocalcisomes (2), we incubated epimastigotes in the presence of these ionophores. However, to preserve their morphology we performed these incubations in culture medium, which contains an appreciable amount of Ca²⁺ (about 1.2 mM), and therefore, we looked for changes in other elements that could indicate the acidic nature of these vacuoles. After 70 min incubation in the presence of ionomycin plus nigericin, there was a marked 3-fold increase in potassium in the electron-dense vacuoles, with no change in the other elements investigated (Fig. 3g and Table II; the potassium content of the calcium-rich vacuoles was about 50% higher in a second experiment, after ionophore treatment; other elements were at similar concentrations in the two experiments, except as noted above for control cells). Since nigericin is a K⁺/H⁺ exchanger this increase in potassium could be due to the vacuoles being acidic. An increase in potassium also occurred in the iron-rich vacuoles (Fig. 3h and Table II) but not in the mitochondria or nuclei. These data suggest that the electron-dense vacuoles and the iron-rich vacuoles were the only acidic compartments of those investigated. The lack of change in calcium concentration in the electron-dense vacuoles could be attributed to refilling by extracellular calcium. The level of cytoplasmic calcium did not change but remained low, implying the operation of calcium pumps (in the vacuoles and in the plasma membrane) even in the presence of ionomycin.
The lack of divalent cation release upon nigericin/ionomycin treatment could also be explained by the higher solubility of divalent cation phosphates at acidic pH. The alkalization of the vacuoles would lead to their intravacuolar precipitation. Since the electron-dense vacuoles are acidic and contain a high calcium concentration, the results suggest that they are the...
were analyzed according to Leapman and Ornberg (26).

The nature of the acidocalcisomes was investigated by separation of cell fractions on Percoll gradients. Ionization edges of phosphorus (K

1–3.) showed vacuoles of similar size and appearance to the electron-dense vacuoles observed in cryosections (Fig. 1) but devoid of electron-dense material or with only some electron-dense material attached to their membranes. After Triton WR-1339 treatment similar vacuoles were observed (Fig. 6b, arrows). However, it is possible that some of the bigger vacuoles not bound by a double membrane might correspond to the electron-dense vacuoles. Separation of extracts of treated cells on Percoll gradients demonstrated varying shifts (decreases) in the density of organelle markers (Fig. 5, stippled bars). Large decreases in the density of the acidocalcisome and the mitochondrion (indicated by succinate cytochrome c reductase activity) were consistently seen in the experiments performed, as were smaller decreases in the density of the other markers. The minor influence on the density of the lysosomal markers, α-mannosidase, acid phosphatase, and cysteine proteinase was surprising, given the original use of Triton WR-1339 as an agent to isolate lysosomes via density reduction (38). The recovery of these enzyme activities was similar from Triton-treated and control cells (average relative recovery of enzymes, treated versus untreated, ranged from 104 to 127%, except for succinate cytochrome c reductase, 82%; this compare with 108% for calcium release from acidic compartments). It was possible that hydrolytic enzymes might also occur in other subcellular locations, particularly the cell surface. Surface location of acid phosphatase and α-mannosidase was tested by comparative assays of whole cells and lysates. An average of only 9% acid phosphatase activity (three experiments, range 4–16%) and 1% α-mannosidase activity (two experiments, both 1%) was on the cell surface; consequently, surface enzyme was not contributing greatly to the observed density profiles of these enzymes.

Labeling of Epimastigotes with Transferrin-Gold—Epimastigotes of T. cruzi bound and internalized gold-labeled transferrin (Fig. 7). Bound transferrin accumulated in an invagination of the plasma membrane, the cytostome, involved in endocytosis (Fig. 7a, c) (39). Internalized transferrin was observed in vacuoles of varying size, some with the heterogeneous content and multivesicular appearance typical of the pre-lysosomal compartment described as the reservosome (Fig. 7b, L), and others with the typical appearance of lysosomes (Fig. 7b, L), but was not observed in the nearly empty vacuoles that corresponded in size to the calcium-containing vacuoles (arrowheads in Fig. 7b; compare with Fig. 6a and Fig. 1). The micrographs shown in Fig. 7 were taken from cells incubated for 1 h with transferrin-gold, but similar results were obtained with cells incubated for 8 h.

**DISCUSSION**

**Elemental Composition of T. cruzi Epimastigotes**—The aim of this study was to characterize morphologically the acidocalcisomes and establish their distinct nature with respect to lysosomes and other intracellular organelles. By using electron

| Compartment                  | Sodium | Magnesium | Phosphorus | Sulfur | Chloride | Potassium | Calcium | Iron | Zinc |
|------------------------------|--------|-----------|------------|--------|----------|-----------|---------|------|------|
| Nucleus (n = 17)             | 59 ± 15| 63 ± 5    | 503 ± 33   | 95 ± 6 | 12 ± 2   | 308 ± 17  | 0.8 ± 0.7|      |      |
| Cytoplasm (n = 19)           | 28 ± 18| 74 ± 9    | 522 ± 35   | 95 ± 6 | 22 ± 5   | 346 ± 23  | 0.3 ± 2.0|      |      |
| Mitochondrion (n = 21)       | 48 ± 9 | 40 ± 3    | 291 ± 30   | 170 ± 10| 21 ± 5   | 225 ± 13  | 1.0 ± 0.6|      |      |
| Kinoplast (n = 14)           | 29 ± 3 | 52 ± 4    | 796 ± 33   | 21 ± 6 | 11 ± 3   | 206 ± 12  | 0.4 ± 0.5|      |      |
| Calcium-rich vacuole (n = 58) | 161 ± 18| 646 ± 19 | 1390 ± 13 | 10 ± 1 | 2 ± 1    | 37 ± 2    | 171 ± 6  | 148 ± 6|      |
| Iron-rich vacuole (n = 11)   | 57 ± 10| 6 ± 2     | 199 ± 9    | 135 ± 11| 183 ± 17 | 72 ± 6    | 0.5 ± 0.6| 128 ± 16|      |

**TABLE I**

Elemental analysis of different compartments in T. cruzi control

Numbers indicate concentration (mean ± S.E.).
probe x-ray microanalysis we found that the only organelles containing a measurable amount of calcium were electron-dense vacuoles. A few previous publications report the use of this technique with trypanosomatids. The earliest study, on *T. cyclops* (9), was not quantitative, but the results are nevertheless similar to those found here; calcium and possibly zinc were detected in the “polyphosphate” granules. The prior work on *T. cruzi* (10), although done on whole cells rather than sections, matches the present data. The only previous analysis of unfixed sections of a trypanosomatid (11) provided data on the elemental composition of calcium-rich vacuoles and cytoplasm in *L. major*. Their results are similar to ours, except that we found chloride levels 4–10-fold lower in both compartments, potassium about 10-fold lower in the calcium vacuoles (untreated cells), and calcium about 10-fold lower in the cytoplasm, and 6–8-fold higher in the vacuoles. The apparent enrichment of calcium in these compartments is therefore much more marked in our study. Vacuoles similar in both appearance and elemental composition occur in the slime mold *Dictyostelium discoideum* (40). The vacuolar content of phosphorus, magnesium, and iron in *T. cruzi* treated with nigericin/ionomycin is shown in Table II.

### Table II

**Elemental analysis of different compartments in *T. cruzi* treated with nigericin/ionomycin**

| Compartment                  | Sodium (mmol/kg dry wt) | Magnesium (mmol/kg dry wt) | Phosphorus (mmol/kg dry wt) | Sulfur (mmol/kg dry wt) | Chloride (mmol/kg dry wt) | Potassium (mmol/kg dry wt) | Calcium (mmol/kg dry wt) | Iron (mmol/kg dry wt) | Zinc (mmol/kg dry wt) |
|------------------------------|--------------------------|----------------------------|-----------------------------|------------------------|--------------------------|---------------------------|--------------------------|----------------------|----------------------|
| Nucleus (n = 17)             | 63 ± 15                  | 68 ± 4                     | 593 ± 4                     | 86 ± 10                | 12 ± 3                   | 292 ± 9                   | 1.5 ± 0.5                | 10 ± 0.9             | 1.3 ± 0.2            |
| Cytoplasm (n = 20)           | 30 ± 14                  | 99 ± 7                     | 579 ± 18                    | 87 ± 4                 | 24 ± 3                   | 346 ± 17                  | 1.2 ± 0.8                | 8 ± 1                | 1.7 ± 0.9            |
| Mitochondrion (n = 21)       | 40 ± 12                  | 37 ± 4                     | 258 ± 15                    | 168 ± 5                | 24 ± 4                   | 228 ± 14                  | 1.7 ± 0.5                | 12 ± 0.8             | 1.7 ± 0.9            |
| Kinetoplast (n = 15)         | 45 ± 15                  | 61 ± 5                     | 818 ± 30                    | 20 ± 5                 | 12 ± 3                   | 179 ± 10                  | 1.3 ± 0.2                | 12 ± 0.8             | 1.7 ± 0.9            |
| Calcium-rich vacuole (n = 63) | 170 ± 12                 | 738 ± 20                   | 1430 ± 14                   | 9 ± 1                  | 2 ± 1                    | 92 ± 7                    | 167 ± 7                  | 131 ± 6              | 1.7 ± 0.9            |
| Iron-rich vacuole (n = 10)   | 35 ± 11                  | 4 ± 3                      | 202 ± 24                    | 145 ± 6                | 161 ± 33                 | 147 ± 26                  | −0.9 ± 1.3               | 182 ± 14             | 1.7 ± 0.9            |

**FIG. 5. Distribution on Percoll gradients of organellar markers from epimastigotes with (stippled bars) or without (shaded bars) Triton WR-1339 treatment.**

The y axis (except for density graph) indicates relative distribution (total = 500 for each gradient); x axis indicates fraction number (1 = lightest). Bars show means from five experiments (four for surface labeling). Density measured in one experiment, using Percoll density marker beads.
and calcium increased substantially during aggregation of slime mold amoebae, passing through the range of concentrations observed here. Zinc was not detected in the slime mold. The sulfur content in these organelles was 10-fold higher than reported here, suggesting a higher protein content. The authors proposed that the phosphate in the vacuoles might be in the form of inositol phosphates, rather than polyphosphate.

EELS measurements on the electron-dense vacuoles indicated the presence of a major organic component. If the vacuolar matrix was composed only of simple phosphates or polyphosphates the expected phosphorus-to-oxygen ratio would be 0.25:1 or 0.33:1, respectively, compared with the measured ratio of 0.11:1 (±15% S.E.). Therefore, at least half of the oxygen comes from organic species. The low measured nitrogen-to-oxygen ratio of 0.065:1 (±13% S.E.) suggests that the major organic component is carbohydrate rather than protein, which is also consistent with the very low sulfur in the electron-dense vacuoles. An additional clue about the composition comes from the fact that the vacuolar matrix “bubbled” under electron irradiation; such bubbling is well-known to occur in sugars (41). It therefore appears likely that a substantial fraction of the vacuolar mass is due to sugar molecules. The small amount of nitrogen detected in the electron-dense vacuoles could originate from membrane proteins included in the analyzed regions. Our x-ray measurements of monovalent (Na+ and K+) and divalent (Mg2+, Ca2+, and Zn2+) cation concentrations allow us to determine the ratio of cation charges per phosphate; the proton concentration is <1mM and can therefore be ignored. From Tables I and II and the requirement of charge neutrality, we find that there are 1.6 ± 0.1 (± S.E.) negative charges per phosphate. This value falls between a polyphosphate composition that would give 1.0 negative charges per phosphate and a pyrophosphate composition that would give 2.0 negative charges per phosphate. Our measurements would be consistent, for example, with sugar diphosphates that have 1.5 negative charges per phosphate. Interestingly, α-glucose 1,2,3,4,6-pentakes -(diphosphate) has recently been identified as a novel metabolite present in phosphorus-, magnesium-, and potassium-containing electron-dense bodies of the sulfate-reducing bacterium Desulfovibrio gigas (42). The nature of the phosphorus-containing metabolite(s) present in T. cruzi epimastigotes is currently being investigated. The presence of calcium-, magnesium-, and phosphorus-containing vacuoles in yeast (43) and possibly also Chlorella (44) further illustrates the importance of these organelles in phylogenetically diverse organisms.

T. cruzi is an obligate intracellular parasite, living in host cell cytoplasm. The high concentration of calcium in an intracellular store could indicate either an adaptation to the cytoplasmic environment where the free Ca2+ concentration is very low (of the order of 10^-7 M) or its requirement for signaling during host cell invasion (1).

Although over 300 enzymes have been identified representing more than 50 different types that are known to require zinc for their function (45), little is known about zinc cell content and free concentration (46). In addition to its accumulation in all trypanosomatids so far examined (see above), zinc is also present in granules in pancreatic beta cells (47) and in certain neurons in the brain, especially in areas of the hippocampus (48), although the reason for its accumulation in any of these compartments is unknown.

A distinctive organelle having an average diameter of 420 ± 70 nm (± S.D.) and much less electron density than the calcium-rich vacuole was found in many cell cross-sections (Fig. 1 and Table I); this organelle was notable for its high iron and chloride content (Fig. 3, f and h). Previously, elemental mapping of whole epimastigotes (10) indicated substantial differences in the iron content of different strains of T. cruzi, which could be reproducibly observed by eye as differences in the color of pellets of cells from the different strains (little iron, white; more iron, brown). T. cyclops contains a prominent iron-containing vacuole (9), visible even with the light microscope although not when it is grown in the absence of hemoglobin.

**Analysis of Acidocalcisomes in T. cruzi**
vacuoles in *T. cruzi* may represent smaller versions of the same, and the iron may be present as degradation products of hemin, an essential component of the epimastigote growth medium. Therefore, these organelles may represent a component of the endo-/lysosomal system in this organism and could correspond to reservosomes (16) that appear as multivesicular bodies using chemical fixation methods (see Figs. 6 and 7). Reservosomes are of similar size to the iron-rich vacuoles and are known to be acidic (49) and to contain cytochrome protease (50). Use of energy-dispersive x-ray microanalysis associated with transmission electron microscopy showed that another trypanosomatid, *Herpetomonas samuellipessoai*, possessed electron-dense granules containing iron (51), similar to those in *T. cyclops* (9).

Treatment of epimastigotes with ionomycin and nigericin markedly increased the potassium content of the electron-dense vacuoles thus indicating their acidic nature. This makes them fulfill the two conditions for the definition of acidocalcisomes, acidity with a high calcium content.

A small but measurable amount of sulfur was found associated with the kinetoplast-DNA (Fig. 3d and Table 1). The ratio of P:S in the kinetoplast was approximately 40:1 compared with about 5:1 in the nucleus. This provides evidence that there is a small amount of protein bound to the kinetoplast-DNA. Although initial cytochemical studies (52) suggested that, like mitochondrial DNA, the kinetoplast-DNA was not associated with proteins, later ultrastructural studies demonstrated their presence (53), and some kinetoplast-DNA-associated basic proteins have recently been identified in *Crithidia fasciculata* (54).

Effects of Triton WR-1339 Treatment on *T. cruzi* Epimastigotes—Triton WR-1339 was the original reagent used to modify the density of lysosomes, and its effects on rat liver were extensively characterized (38). It was also injected into rats infected with *T. brucei* (55), where it produced large vacuoles in the trypanosomes. Parasite proteinase activity was largely recovered from density gradients in the position of cytosolic proteins, suggesting the lysosomal membrane had been weakened by the treatment, although there was only a small decrease in the density of recovered α-mannosidase activity. The position of non-lysosomal enzymes in density gradients was not reported (55). We found, in *T. cruzi* epimastigotes, no change in the density of lysosomal markers (and no reduction in the yields of any marker enzymes) following Triton WR-1339 treatment. These data imply that the lysosomal effects are dependent upon uptake of the detergent by endocytosis. Bloodstream forms of *T. brucei*, as well as having well-characterized receptor-mediated up takes mechanisms for transferrin and low density lipoprotein, also readily take up markers for fluid-phase endocytosis (56, 57). Epimastigotes will take up gold-labeled proteins including low density lipoproteins. These results have been used as evidence that receptor-mediated endocytosis is occurring (49), but the uptake of catalase was shown to be a non-saturable process, indicating that membrane receptors were not involved (39). Receptor-mediated endocytosis (of transferrin) has been demonstrated biochemically only in the amastigote stage of *T. cruzi* (58). We confirmed that epimastigotes take up gold-labeled transferrin (Fig. 7), but the extent of uptake was insufficient to modify the lysosome density, as achieved with lymphocytes (59). Similarly, we were unable to increase the density of lysosomes by dextran loading (28, 60). We conclude, therefore, that *T. cruzi* epimastigotes are not very efficient at endocytosis and that the observed effects of Triton WR-1339 may arise via a route other than endocytosis; its effects are therefore not restricted to lysosomes/endosomes. Electron microscopy showed that vacuolation in epimastigotes was most prominent in mitochondrial elements (shown by the presence of the kinetoplast-DNA in vacuoles or vacuoles with double limiting membranes). However, the detergent’s density-altering effects were not necessarily limited to the mitochondrion, and the separation of mitochondria from acidocalcisomes was shown by the following: 1) the discrepancy between Percoll gradient profiles of “acidic calcium” and succinate cytochrome c reductase activity recovered from Triton-treated cells; 2) the assays for acidic calcium were performed in the presence of 1 μg/ml antimycin A and 2 μg/ml oligomycin, which will deplate mitochondria of calcium (61); and 3) mitochondria are not acidic (62), so any calcium therein should be releasable by ionomycin, which was preincubated with fractions prior to assay for acidic calcium storage.

**Distinguishing Acidocalcisomes from Lysosomes**—The above results suggest that the acidocalcisome is an organelle distinct from the lysosome in *T. cruzi* epimastigotes. In mammalian cells Ca\(^{2+}\) has also been reported to be present in organelles having an acidic milieu, such as endosomes, lysosomes, the trans-Golgi network, and secretory granules including chromaffin, pancreatic zymogen, and atrial-specific granules (62–67), but the functional significance of the high Ca\(^{2+}\) content of these organelles is unknown (62). In addition, a Ca\(^{2+}\)-ATPase from rat liver lysosomes has been characterized (68), and a Ca\(^{2+}\)-ATPase gene that was cloned from rat stomach (69) exhibits 50% amino acid identity with the Golgi-located *PMR1* gene of *Saccharomyces cerevisiae* (70). Calcium storage has also been reported in lysosomes in *Trypanosoma rhodesiense* (71). To establish further the distinction between acidocalcisomes and lysosomes, we loaded epimastigotes with gold-labeled transferrin to identify the endosomal/lysosomal system in the parasites (Fig. 7). Internal compartments were clearly labeled, but these were distinct from the vacuoles that corresponded to the electron-dense organelles (Fig. 7 and Fig. 6a). The report on *T. rhodesiense* (71) was based on the apparent coincidence of calcium storage with acid phosphatase location. Electron-dense vacuoles were identified as “lysosomal vesicles,” but the basis for this designation was not established. A possible acid phosphatase reaction was shown in one vacuole, but adequate controls were not presented. Additionally, the presence of acid phosphatase in an organelle does not mean it is a lysosome. Williamson and McLaren (71) themselves showed activity in the flagellar pocket of *T. rhodesiense*, whereas previous subcellular fractionation of *T. cruzi* (Tulahuen strain) indicated a likely non-lysosomal location of acid phosphatase (30). The activity has been also detected cytochemically in the Golgi and on the cell surface of *T. cruzi* (39). We found a minor component (9%) of the activity associated with the cell surface in the epimastigotes that we used. As indicated above, the elemental composition of *L. major* electron-dense vacuoles (11) is quantitatively different from that of the *T. cruzi* acidocalcisomes reported here, and we cannot rule out a different biochemical composition in acidocalcisomes of different stages of *T. cruzi* or in the different parasites in which they have been described (2–7).

In conclusion, we found evidence to suggest that the electron-dense vacuoles previously detected in *T. cruzi* epimastigotes (10) correspond to what has been described biochemically as acidocalcisomes (2), as indicated by their high calcium content and their acidic character revealed by their sensitivity to nigericin treatment. In addition, cell fractionation studies and gold-labeled transferrin experiments indicated that these organelles are different from lysosomes or other endocytic vacuoles. The presence in trypanosomatids of a distinct organelle with apparently no counterpart in mammalian cells makes it a potential target for chemotherapy.
