Table I. Digestion experiments with pancreatic elastase

Conditions adopted	Enzyme activity (U/mg)
0.01 *M* Tris + 0.1 *M* KCl; pH 8.0	
a) 25°C	5.3
b) 37°C	7.6
c) 37°C; 2 *M* urea	5.0

Protein concentration: 0.8 mg/ml. Ratio toxin to elastase: 10/1. Incubation time: 180 min.

Table II. Digestion experiments with papain

Conditions adopted	Enzyme activity (U/mg)
0.4 *M* NaCl; 37°C	
a) pH 8.0	2.8
b) pH 8.0; 2 *M* urea	2.0

Protein concentration: 0.8 mg/ml. Ratio toxin to papain: 100/1. Incubation time: 180 min.

showed a main band, accounting for most of the protein content; the toxic activity, as determined by the 'blueing doses' assay (skin test) in the rabbit, was 700,000 blueing doses per mg of protein.

The digestions were performed at constant pH, under nitrogen in a Combi-Titreur apparatus (Metrohm AG, Switzerland); the alkali uptake (index of enzymatic digestion) was recorded automatically. Pancreatic elestase (3.4.4.7, Serva, Germany) and Papain (3.4.4.10, Type II, Sigma, USA) were titrated with the synthetic substrates N-benzoyl-L-alanine methylester (BAME[5]) and N-benzoyl-L-arginine ethylester (BAEE[6]), respectively, before their use in the digestion experiments. The conditions adopted for the enzymatic treatments are shown in Tables I and II.

Results and discussion. None of the digestion schemes adopted showed evidence of alkali uptake during the treatment of the toxin with either pancreatic elastase or papain. On the other hand, it was possible to evidence complete activity of the enzymes at the end of each single digestion experiment by titrating an aliquot of the digestion mixtures with the specific synthetic substrates.

Samples of the toxin incubated with pancreatic elastase at 25° and 37°C were also tested in order to assay the effect of the enzymatic incubation on the electrophoretic pattern, agar immunodiffusion and biological toxic activity. The toxin did not show any changes in these parameters, which were identical to those found in the toxin samples maintained under the same experimental conditions but in the absence of the enzyme (blank experiments). On the other hand, the enzymes showed no effects in any of the tests.

The blank experiments for the samples incubated in 2 *M* urea showed a partial modification of the properties of the toxin; this denaturation, however, was not sufficient to allow the Choleragen to be attacked by the two enzymes.

The results of these experiments show that Choleragen, in the conditions adopted, is resistant to the action of pancreatic elastase and papain, and they support the hypothesis of a particular molecular structure as an explanation of the activity of the toxin in the intestinal lumen.

More detailed studies regarding enzymatic digestion of Choleragen, also in the presence of denaturants, could clarify the relationship between molecular structure and mechanism of action of the toxin.

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Localization of Peroxidase Activity in *Trypanosoma cruzi* Microbodies[1]

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Summary. Electron microscopic observation of *Trypanosoma cruzi* epimastigotes reveals the presence of microbody-like structures (microperoxisomes) in which 3,3′-diaminobenzidine (DAB) is peroxidized to electron-opaque material. The role of peroxidase in DAB peroxidation is supported by the enzyme demonstration in disrupted epimastigotes and the microbody-containing cell fractions.

Microbodies ('peroxisomes', 'microperoxisomes') are defined as cytoplasmic structures characterized by the association of one or more hydrogen peroxide-producing oxidases with catalase, which destroys the hydrogen peroxide[4–7]. The organelles are recognized as ubiquitous structures in living cells[8], including protozoa[9]. In the bloodstream, forms of *Trypanosoma cruzi* (the agent of Chagas disease), oval bodies have been postulated to be peroxisomes[10]. Incubation of aldehyde-fixed cells in alkaline 3,3′-diaminobenzidine (DAB) media is a suitable procedure for demonstrating peroxisomes since DAB peroxidation determines the formation of electron-opaque material, easily visualized by electron microscopy[11,12]. In this paper we demonstrate the existence of DAB positive, microbodylike structures (microperoxisomes) in the epimastigote (culture) form of *T. cruzi*.

Materials and methods. The Tulahuen strain of *T. cruzi* was grown in a diphasic medium at 28°C, as described before[13]. 4 days after inoculation, the cells were collected and reinoculated in a liquid medium made of NaCl (9 g); Na_2HPO_4 (7.5 g); KCl (0.4 g); glucose (4.0 g); tryptose (Difco; 15 g); yeast extract (Difco; 5 g); liver extract (5 g); inactivated calf serum (5 ml); hemin (Sigma Chemical Company; 20 mg, dissolved in 8 ml of 0.1 *N*

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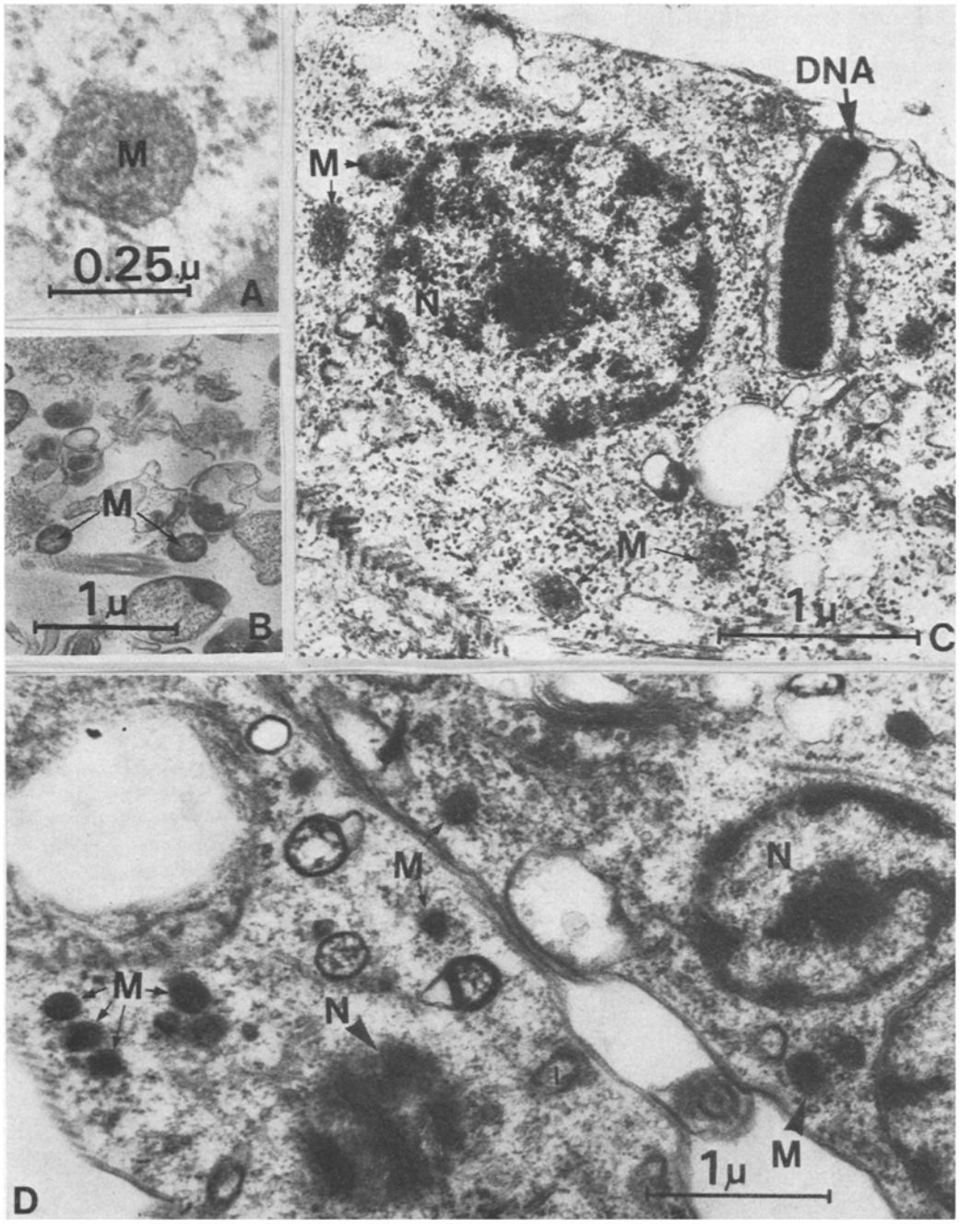

Fig. 1. A) microbody (M) in *T. cruzi* epimastigote. Section fixed with glutaraldehyde, postfixed with osmium tetroxide and counterstained with lead citrate. B) same in the $480 \times g$ fraction (fractionation as described in the Table). C) sections of epimastigotes previously stained with DAB for peroxidase activity (control specimen; hydrogen peroxide omitted). D) same as C) hydrogen peroxide added. Note that dark microbodies stand out against the other organelles. M, microbodies; N, the nucleus; DNA, the kinetoplast. Other experimental conditions were as described in the text.

NaOH containing 3.7 g triethanolamine-HCl); and water (1 l). The liquid medium was maintained in a water-bath at 28 °C with constant shaking. 6 days after inoculation, epimastigotes were collected by centrifugation at $3000 \times g$ for 10 min and washed 3 times in the centrifuge with isotonic (0.154 *M*) NaCl solution.

For electron microscopy, epimastigotes were fixed in 3% glutaraldehyde in 0.1 *M* potassium phosphate buffer (pH 7.2), for 1 h, at 22–24 °C, and postfixed in 1.5% (w/v) osmium tetroxide in 0.1 *M* phosphate buffer, in the cold. After dehydration with ethanol, the material was embedded in Epon, sections were cut with a Porter-Blum ultramicrotome, stained with Reynolds' lead citrate, and observed in a Siemens Elmiskop 101, as described in ref.[14].

Staining with DAB. Glutaraldehyde-fixed epimastigote were incubated with the DAB medium (prepared immediately before use) that contained DAB (Sigma Chemical Company; 20 mg), 0.05 *M* propanediol buffer, pH 10; 1% H_2O_2 (0.2 ml) and 0.22 *M* sucrose (total volume, 10 ml)[15]. The pH was adjusted to 9.0 prior to addition of the epimastigotes. After 60 min incubation, the epimastigotes were washed several times, for 15–20 min, with 0.3 *M* sucrose–0.1 *M* phosphate buffer (pH 7.2). Postfixation, dehydration, embedding and sectioning were as described above.

Peroxidase activity in *T. cruzi* fractions[a]

Fraction	Peroxidase specific activity[b] (nmol ascorbate oxidized/min/mg protein)
Homogenate	15.8
$480 \times g$	41.2
$680 \times g$	26.2
$12{,}000 \times g$	4.4
$105{,}000 \times g$	4.5
Supernatant	5.7

[a] The isolation medium was made of 0.25 *M* mannitol, 10 m*M* morpholinopropane sulfonic acid; 2.0 g/l polyvinylpyrrolidone, and 3.0 g/l bovine serum albumin (fraction V, Nutritional Biochemicals Corp.); 4.5 m*M* ascorbate, 0.25 m*M* $MgCl_2$ and 0.25 m*M* EDTA; pH 8.0. Digitonin in dimethylformamide 40 mg/ml) was added to provide 6.0 mg/g cells.
[b] Peroxidase assay was performed as described in Figure 2: 0.03–0.25 mg of protein/ml.

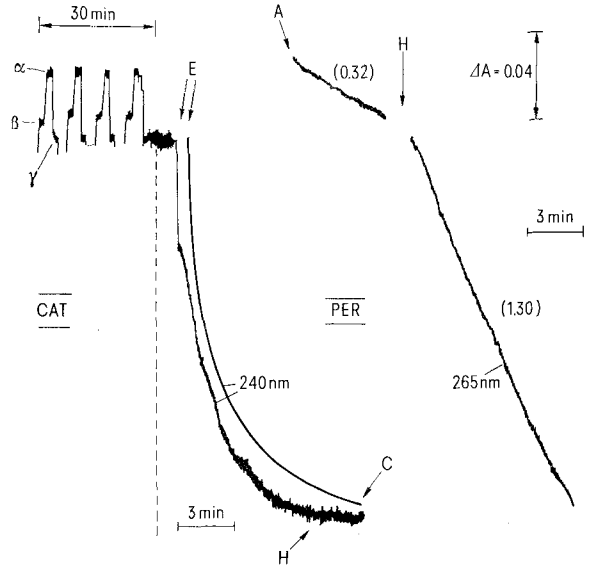

Fig. 2. Catalase and peroxidase assays in *T. cruzi* homogenates. Cells disrupted by freezing and thawing.

Fractionation of epimastigotes was performed after cell disruption in a Polytron blendor (Bronwill Scientific Company), as described by KUSEL and STOREY[16]. For catalase and peroxidase assays, epimastigotes were disrupted by freezing (at −16 °C) and thawing 3 times. The cell suspension (0.1–0.2 ml) was homogenized by several passages through a narrow hypodermic needle, attached to a syringe. Catalase activity was determined spectrophotometrically, by the decrease in absorbance of the hydrogen peroxide containing reaction mixture at 240 nm[17]. Peroxidase activity was determined using ascorbic acid (Sigma Chemical Company) as hydrogen donor[18] and measuring its oxidation by hydrogen peroxide at 265 nm ($E_{mM} = 16\ cm^{-1} \cdot mmol^{-1}$).

Catalase assay (CAT; left tracing). The reaction mixture contained 50 m*M* phosphate buffer, pH 7.3, 2.0 m*M* H_2O_2 and epimastigote homogenate (0.48 (α); 0.24 (β) and 0.12 (γ) mg protein/ml); final vol. 3 ml. Samples were placed in the spectrophotometer cuvette positioner and automatically scanned at 240 nm, for 30 min. 2 initial and 2 final tracings are shown in Figure 2. Where indicated by the arrow E, beef-liver catalase (10 µl) was added to sample (final hematin concentration, 0.6 n*M*). The downward deflection indicates the catalatic decomposition of H_2O_2. A control (C) reaction mixture, except for homogenate (H) was added catalase as above. Note the similarity of tracings H and C.

Peroxidase assay (PER; right tracing). The reaction mixture contained 50 m*M* *Tris*-HCl buffer, pH 7.3, 1 m*M* EDTA, 54 µ*M* Na ascorbate (A) and epimastigote homogenate (H) (0.48 mg protein/ml) added where indicated by arrows A and H, respectively; final vol. 3 ml. The figures in parenthesis indicate ascorbate disappearance (µ*M*/min). The 0.32 value represents the rate of ascorbate autoxidation. Omission of H_2O_2 determined that the rate of autoxidation did not increase after homogenate addition. Variations of absorbance were recorded in the Gilford Model 2000 spectrophotometer, at 30 °C. Other experimental conditions were as described in the text. The absorbance scale is valid for catalase and peroxidase tracings.

Results. Figure 1A and B show microbodies about 0.15–0.10 µm as they typically appear in epimastigote sections (A) (or fractions (B)) fixed with glutaraldehyde, postfixed with osmium tetroxide and counterstained with lead citrate. Incubation of glutaraldehyde-fixed epimastigotes in media containing DAB and hydrogen peroxide resulted in pronounced deposition of electron-opaque material within structures clearly recognizable as microbodies (Figure 1D). The enhanced electron opacity results from the deposition of a finely granular product of DAB peroxidation. It is remarkable that DAB peroxidation did not occur in the kinetoplast mitochondrion, or in the general cytoplasmic matrix (Figure 1D). Omission of hydrogen peroxide from the reaction mixture significantly reduced the staining of microbodies (Figure 1C).

Since peroxidation of DAB is interpreted as a demonstration of the presence of catalase[11,19–21], the enzyme

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was directly investigated in disrupted epimastigotes. Figure 2 shows, however, that no catalase activity could be detected in the epimastigote homogenate. The possible existence of catalase inhibitors in the homogenate may be excluded by comparing the activity of beef liver catalase (Sigma Chemical Company; C-100) in homogenate-containing and homogenate-free reaction mixtures, respectively (Figure 2). In contrast to these negative results, peroxidase activity could be demonstrated in the epimastigote homogenate (Figure 2) and also in the particulate fractions obtained therefrom (Table). Peroxidase activity was closely associated with the particulate $480 \times g$ and $680 \times g$ fractions, which included the microbodies (Figure 1B). The non-sedimentable fraction at $105{,}000 \times g$ showed a significant, though lower, peroxidase-specific activity, which might reflect the release of enzyme from microbodies broken during cell fractionation. In the absence of ascorbate, the rate of hydrogen peroxide decomposition by the microbody-containing fractions was negligible (0–3% of the rate in the presence of ascorbate).

Discussion. DAB positivity is standard cytochemical evidence for the identification of catalase-containing microbodies[4–6, 19, 20] but the reaction is also positive with peroxidase[21–23] and cytochrome oxidase[12, 21, 24]. The presence of peroxidase in *T. cruzi* microbodies is in good agreement with a) the preferent distribution of peroxidatic activity in the parasite high density fractions (Table); b) the fact that peroxidases selectively oxidize donors having the enediol structure, such as ascorbate[18] (Figure 2 and Table), and c) the cytochemical demonstration of peroxidase in *T. cruzi* by KALLINICOVA[25]. Participation of cytochrome oxidase in DAB peroxidation may be excluded by the restricted distribution of the electron-opaque material in Figure 1. The apparent absence of catalase in *T. cruzi* microbodies recalls similar negative observations with microbodies from *Trichomonas foetus*[26]. Furthermore, investigation of catalase in other *Trypanosomatidae*[27–29] and *Trichomonas*[30], with methods different from the one employed by us, failed to demonstrate significant amounts of enzyme in these organisms. *T. brucei* has no catalase, but shows peroxidase activity[9].

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Electron-Microscopic Mapping of the Hinge Region of Myosin

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Summary. The trypsin-sensitive sites in the labile hinge region of the myosin molecule are located with heightened accuracy ($\pm$ 2 nm) by electron microscopy as lying at 70, 85, 95, and 103 nm from the C-terminus of the rod section of the molecule.

A restricted region within the rod section of the myosin molecule lying at 70–110 nm from the C-terminus shows special properties including high susceptibility to proteases[3–7] and to thermal denaturation[8]. It has been termed the *hinge* region, and a role in muscle shortening has been proposed[8–10]. The loci must susceptible to tryptic attack have been inferred from the molecular weights of digestion products or determined in the electron microscope[7, 11, 12]. I report here a more accurate mapping of the sites of tryptic attack on myosin that has been aggregated into segment structures or on similar arrays of light meromyosin-C (LMM-C), which is a C-terminal fragment of myosin that is liberated by digestion with BrCN[13], and which contains most of the hinge region. Advantageously, the molecules in the planar arrays are held straight by contact with neighbors, and positions of digested margins can be accurately measured in the electron microscope.

Myosin and LMM-C were prepared[13–15] and aggregates were grown by dialysis-dilution[16] to a final concentration of 0.22 *M* calcium acetate. The resulting myosin aggregates were aligned segments[16], and those with LMM-C were a segment structure, phase F′, with an overlap width of 88 $\pm$ 4 nm and a fringe width of 12 $\pm$ 2 nm (all limits of error are standard deviations). LMM-C was also aggregated by dialysis-dilution from 0.7 *M* to 0.5–0.59 *M* calcium butyrate to give a new segment structure, phase R, with an overlap width of 64 $\pm$ 2 nm and a fringe width of 30 $\pm$ 1 nm.

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