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The detection of phosphonolipids in the protozoan *Trypanosoma cruzi*

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2-Aminoethylphosphonate was detected in the acid hydrolysates of the phosphonolipids and the lipopeptidophosphoglycan of *Trypanosoma cruzi*, the causative agent of Chagas' disease. This finding represents the first evidence of phosphonolipids in a zooflagellate. By comparison, no phosphonolipids were detected in *Trypanosama brucei*, indicating that phosphonolipids are not a ubiquitous feature of the Order Kinetoplastidia.

Trypanosoma cruzi, a parasitic protozoan of the Order Kinetoplastidia, is the organism responsible for South American trypanosomiasis (Chagas' disease). *T. cruzi* has a complex host–vector life-cycle, existing in the mammalian host as an intracellular dividing form (amastigote) and as a non-dividing bloodstream form (trypomastigote). In the insect vector it exists in the gut as a dividing form (epimastigote), and the infection is transmitted to the mammalian host via the non-dividing metacyclic form in the insect faeces.

Phosphonolipids are phospholipids with a direct bond between the phosphorus atom and a carbon atom of the nitrogenous base in the place of the normal phosphoester linkage. Their occurrence and biochemistry have been reviewed by Rosenberg (1973). Phosphonolipids have been detected in endogenous lipids of eukaryotic cells in the Phyla Protozoa, Coelenterata and Mollusca. Within the Phylum Protozoa phosphonolipids have so far been found in ciliates (Liang & Rosenberg, 1966; Dawson & Kemp, 1967), in amoebae (Korn *et al.*, 1974) and in phytoflagellates (H. Rosenberg, personal communication). We now report here the presence of low concentrations of 2-aminoethylphosphonate in the acid hydrolysates of both *T. cruzi* epimastigote lipids and the epimastigote lipopeptidophosphoglycan (De Lederkremer *et al.*, 1977, 1978), which is a cell-surface glycoconjugate. No 2-aminoethylphosphonate was detected in similar studies with the African trypanosome *Trypanosoma brucei*.

Experimental

Parasite culture

T. cruzi epimastigotes were a clone (Well. Tryp. Y_2C_1) derived from the Y strain. Epimastigotes were grown in culture at 25°C in the medium of Boné & Parent (1963) containing 5% (v/v) rabbit serum, penicillin (200 μg/ml) and streptomycin (100 units/ml). Biosynthetic radiolabelling with ^{32}P was performed by incubating 10^8 epimastigotes in 5 ml of RPMI 1640 medium (Flow Laboratories, Irvine, Scotland, U.K.) containing 5% (v/v) foetal calf serum with 1 mCi of $^{32}P_i$ (Amersham International) at 25°C for 24 h. After incubation the cells were washed twice in phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2) at 4°C.

T. brucei procyclics (insect gut form) were strain S42, isolated from an adult female warthog (*Phacochoerus aethiopicus* Pallus) in Tanzania by Dr. J. R. Baker. They were cultured in the medium of Brun & Schonenberger (1979).

T. brucei trypomastigotes (host bloodstream form) were a clone (MIT at 1.4) derived from strain 427 (Cross, 1975), and were prepared from infected rat blood by the method of Lanham & Godfrey (1970).

Preparation and processing of extracts

Total lipid extracts were obtained by extracting cell pastes three times with 20 vol. of chloroform/methanol (2:1, v/v). Extracts were washed three times with water and dried under a stream of N_2.

The lipopeptidophosphoglycan, which is insoluble in chloroform/methanol, was prepared from *T. cruzi* epimastigotes by phenol extraction followed by chloroform/methanol/water extraction by the method of De Lederkremer *et al.* (1977).

Acid hydrolysis of the lipid preparations was performed in 6 M-HCl for 48 h at 120°C in sealed tubes. Under these vigorous conditions all phosphoester and diester bonds are cleaved to yield P_i; by contrast, the highly stable C–P bond of phosphonyl compounds is conserved (Liang & Rosenberg, 1966; Rosenberg, 1973). The hydrolysate of the ^{32}P-labelled epimastigote lipids was dried *in vacuo* over

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P_2O_5 and NaOH and redissolved in a small volume of water for subsequent analysis for 2-aminoethylphosphonate. The hydrolysates of the lipopeptidophosphoglycan and bulk *T. cruzi* and *T. brucei* lipid preparations were extracted three times with hexane to remove released fatty acids and filtered through Whatman GF/F paper to remove charred products. 2-Aminoethylphosphonate in these samples was enriched by passing the neutralized hydrolysate through a column of Dowex 1 X2 (acetate form) (Liang & Rosenberg, 1966). This procedure allows the elution of most of the amino acids with water and gives quantitative yields of 2-aminoethylphosphonate when the column is eluted with 5% (v/v) acetic acid. Bound P_i from ordinary phospholipids was quantitatively removed by subsequent elution with 2M-HCl. The 2-aminoethylphosphonate fractions were dried and redissolved in a small volume of water for analysis.

Analytical methods

2-Aminoethylphosphonate was identified by high-voltage paper electrophoresis (Atfield & Morris, 1961) on Whatman 3MM paper in 0.73M-acetic acid/0.27M-formic acid buffer, pH 1.9, at 100V/cm for 40min on a Shandon cooled plattern apparatus. 2-Aminoethylphosphonate was detected by spraying electrophoretograms with ninhydrin/cadmium reagent (Atfield & Morris, 1961) and heating at 80°C for 30min. ^{32}P-labelled 2-aminoethylphosphonate was detected by radioautography at −70°C with Kodak X-omat film and Kodak X-omat regular intensifying screens.

2-Aminoethylphosphonate was determined quantitatively by using a Locarte Mini amino acid analyser; the 25cm × 0.9cm column was successively eluted with 0.2M-sodium citrate buffer, pH 2.6, for 120min and 0.2M-sodium citrate buffer, pH 4.4, for 180min, at a flow rate of 30cm/h at 50°C. The internal standard was *p*-fluorophenylalanine. Organic phosphate was assayed by the method of Bartlett (1959).

Results

Phospholipids of *T. cruzi* were biosynthetically labelled with ^{32}P for 24h, extracted into chloroform/methanol and hydrolysed with 6M-HCl under vigorous conditions. The hydrolysate was examined by high-voltage paper electrophoresis. The radioautograph (Fig. 1) of the electrophoretogram showed two radioactive components that had migrated from the origin, one a fast-moving heavily labelled anion and the other a slow-moving cation. The former corresponded to P_i derived from ^{32}P-labelled phospholipids, and the slow-moving cation was shown to co-migrate with an authentic 2-aminoethylphosphonate standard. This result establishes the presence of low concentrations of phosphonolipids in *T. cruzi* epimastigotes.

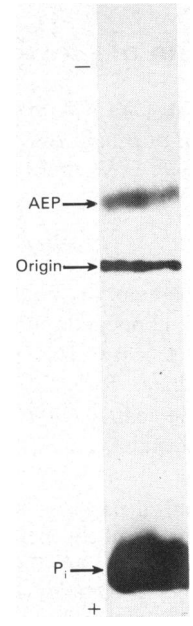

Fig. 1. *Radioautograph of the electrophoretogram of the acid hydrolysate of biosynthetically ^{32}P-labelled phospholipids of T. cruzi*

For experimental details see the text. The slow-moving cation was shown to co-migrate with an authentic 2-aminoethylphosphonate (AEP) standard.

The electrophoretograms of the 2-aminoethylphosphonate fractions from the acid hydrolysates of bulk lipid preparations of *T. cruzi* epimastigotes and *T. brucei* procyclics are shown in Fig. 2. The result again demonstrates the presence of 2-aminoethylphosphonate in phosphonolipids in *T. cruzi* epimastigotes, but shows a definite lack of 2-aminoethylphosphonate in *T. brucei* procyclics. (Similar studies also failed to find evidence for 2-aminoethylphosphonate in the bloodstream form of *T. brucei*; results not shown.) The 2-aminoethylphosphonate in these fractions was determined quantitatively by using the amino acid analyser system. These results for phosphonyl phosphorus are compared with the values for phosphoryl phosphorus recovered from the Dowex columns (Table 1).

The *T. cruzi* epimastigote lipopeptidophosphoglycan was also shown to contain 2-aminoethylphosphonate. Fig. 3 shows the presence of a faint ninhydrin-positive band from the lipopeptidophosphoglycan hydrolysate co-migrating with an authentic 2-aminoethylphosphonate standard. The results of quantitative analysis for 2-aminoethylphosphonate and phosphate in this material are given in Table 1.

Table 1. *Comparison of phosphonate contents of T. brucei and T. cruzi lipid fractions*
For experimental details see the text.

Material analysed ...	*T. brucei* procyclic lipid	*T. cruzi* epimastigote lipid	*T. cruzi* lipopeptidophosphoglycan
Amount of material hydrolysed (mg dry wt.)	21	16	2
Phosphoryl phosphorus content (nmol/mg)	648	481	575
Phosphonyl phosphorus content (nmol/mg)	<0.05	1.66	36.0
Phosphonyl phosphorus content (% of total phosphorus content)	<0.01	0.34	5.9

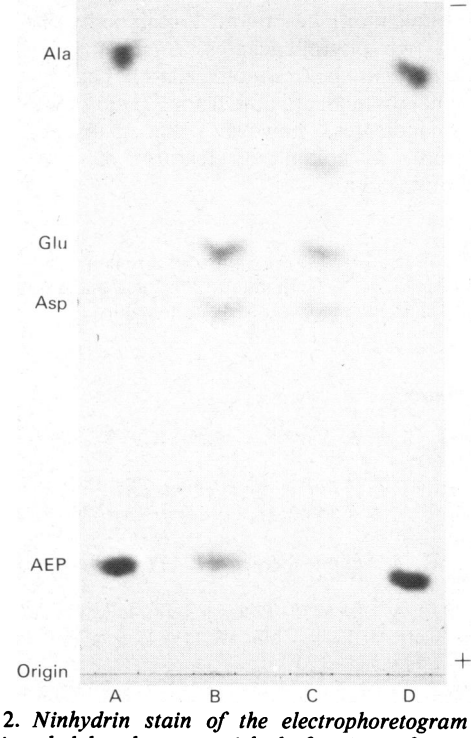

Fig. 2. *Ninhydrin stain of the electrophoretogram of 2-aminoethylphosphonate-enriched fractions from the acid hydrolysates of bulk lipid extracts*
For experimental details see the text. Track B, *T. cruzi* epimastigote lipid (3.2mg); track C, *T. brucei* procyclic lipid (4.2mg); tracks A and D, authentic 2-aminoethylphosphonate (AEP) and alanine standards.

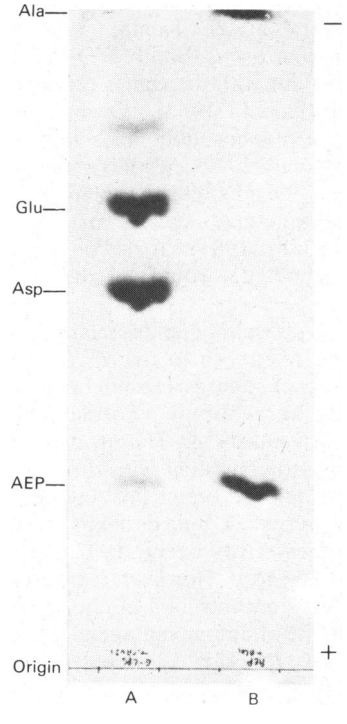

Fig. 3. *Ninhydrin stain of the electrophoretogram of the 2-aminoethylphosphonate-enriched fraction from the acid hydrolysate of T. cruzi lipopeptidophosphoglycan*
For experimental details see the text. Track A, *T. cruzi* lipopeptidophosphoglycan (200 μg); track B, authentic 2-aminoethylphosphonate (AEP) and alanine standards.

To verify the identification of 2-aminoethylphosphonate, a sample of lipopeptidophosphoglycan hydrolysate was subjected to high-voltage paper electrophoresis and stained lightly with ninhydrin, and the putative 2-aminoethylphosphonate band was cut out and eluted with water. This solution was run on the amino acid analyser system used for quantification. One ninhydrin-positive component, with the same retention time as authentic 2-aminoethylphosphonate, was observed. The identification of 2-aminoethylphosphonate by these two independent methods firmly establishes its identity.

Discussion

Previous reports have excluded the possibility of alkylphosphonic acid as a component of the lipopeptidophosphoglycan (De Lederkremer *et al.*, 1976) and indicated an absence of phosphonolipids from *T. cruzi* epimastigotes (Da Silveira & Colli, 1981). However, both of these statements were made on the basis that their results for total phosphorus and acid-hydrolysable phosphorus were in close agreement, This would indeed be the case, as

phosphonates are present only in very low concentrations in *T. cruzi* lipids and lipopeptidophosphoglycan and are probably only detectable by positive identification, as done in the present study. Da Silveira & Colli (1981), however, do present some indirect evidence for the existence of other phosphonate-containing molecules in lipid-free membrane preparations from *T. cruzi* epimastigotes.

The phospholipids of *T. cruzi* represent only 0.34% of the total phospholipid population. This concentration is very low compared with those reported in other protozoa [cf. 24% in rumen protozoa (Dawson & Kemp, 1967), 23% in *Tetrahymena pyriformis* (Smith & O'Malley, 1978)], and is more in line with the values reported for some bacteria (Horiguchi, 1966). Owing to the low concentrations of phosphonolipids in *T. cruzi*, the actual phosphonolipid species have not been identified, but, as phosphatidylethanolamine is a predominant phospholipid species in *T. cruzi* (Da Silveira & Colli, 1981), it is possible that its phosphonyl analogue would be the major phosphonolipid.

The role of phosphonolipids is largely unknown, although their resistance to hydrolytic enzymes, in particular phospholipase D, might confer some advantage to an organism in a hostile or competitive environment (Kennedy & Thompson, 1970). This hypothesis is also consistent with the fact that, with very few exceptions, organisms containing phosphonolipids present a naked living membrane to their environment (Rosenberg, 1973), and *T. cruzi* falls into this category. However, it is difficult to see how such a low concentration of phosphonolipids in *T. cruzi* could be of any advantage in this context. It might be that *T. cruzi* is weakly expressing a characteristic inherited from a more primitive free-living ancestor, the evolutionary advantage of having phosphonolipids having been lost when it adopted its host–vector life-cycle.

The total absence of phosphonolipids from *T. brucei* procyclics is not particularly surprising. *T. cruzi* and *T. brucei* exist in geographically distinct regions, and it is generally accepted that, although they are probably derived from a common free-living ancestor, the two subgenera to which they belong probably diverged very early and evolved separately (Lumsden, 1974).

The presence of 2-aminoethylphosphonate as a component of the lipopeptidophosphoglycan is a finding worthy of note. Although the small quantities of 2-aminoethylphosphonate in this compound probably preclude it from being an obligatory component, it might be inferred that its phosphoryl analogue, phosphoethanolamine, is an obligatory component. A potentially similar molecule, the lipophosphonoglycan from *Acanthomoeba castellanii*, has also been shown to contain 2-aminoethylphosphonate and 2-amino-1-hydroxyethylphosphonate (Korn *et al.*, 1974; Dearborn *et al.*, 1976), although in much greater amounts (10% aminophosphonates). However, the relative enrichment of phosphonyl to phosphoryl phosphorus in the lipopeptidophosphoglycan as compared with the phospholipids of *T. cruzi* might suggest some structural advantage of C–P bonds in these complex protozoal glycophospholipids.

This is the first evidence for phosphonolipids in a zooflagellate, although H. Rosenberg (personal communication) has found 2-aminoethylphosphonate in two phytoflagellates, *Euglena gracilis* and *Amphidinium* sp. (a dinoflagellate). The absence of 2-aminoethylphosphonate from *Trypanosoma brucei* lipids indicates, however, that phosphonolipids are not a ubiquitous feature of the Order Kinetoplastidia.

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