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^{31}P NMR Spectroscopy of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*

EVIDENCE FOR HIGH LEVELS OF CONDENSED INORGANIC PHOSPHATES*

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High resolution ^{31}P nuclear magnetic resonance spectra at 303.6 MHz (corresponding to a ^{1}H resonance frequency of 750 MHz) have been obtained of perchloric acid extracts of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*, the causative agents of African sleeping sickness, Chagas' disease, and leishmaniasis. Essentially complete assignments have been made based on chemical shifts and by direct addition of authentic reference compounds. The results indicate the presence of high levels of short chain condensed polyphosphates: di-, tri-, tetra-, and pentapolyphosphate. ^{31}P NMR spectra of purified *T. brucei*, *T. cruzi*, and *L. major* acidocalcisomes, calcium and phosphorus storage organelles, indicate that polyphosphates are abundant in these organelles and have an average chain length of 3.11–3.39 phosphates. In the context of the recent discovery of several pyrophosphate-utilizing enzymes in trypanosomatids, the presence of these inorganic polyphosphates implies a critical role for these molecules in these parasites and a potential new route to chemotherapy.

Infections caused by trypanosomatid parasites continue to be among the most widespread diseases in developing nations, and the increasing fraction of immunocompromised individuals in the global population further contributes to morbidity and economic loss. New chemotherapeutic leads based on unique aspects of parasite biology are therefore of great interest, particularly in the case of emerging resistance to traditional treatments.

One approach to the discovery of new drug targets is to identify metabolic pathways in parasites that are essential for parasite survival but do not have an equivalent in the host. Then, one can pursue specific inhibitors of such metabolic activities as possible means of controlling the parasites without damaging the host. NMR spectroscopy has already been used by several groups to investigate the carbohydrate and/or phosphate metabolism of protozoa, including *Crithidia fasciculata* (1), *Entamoeba histolytica* (2), *Trypanosoma cruzi* (3, 4), *Trypanosoma brucei* (5, 6), *Leishmania pifanoi* (7), *Babesia microti* (8), and the free-living ciliate *Tetrahymena pyriformis* (9). In *Tetrahymena*, large levels of pyrophosphate were found, and more recently we have found similarly large levels of pyrophosphate in *T. cruzi* (10). These observations are of interest since a number of protozoa are already known to utilize pyrophosphate in addition to ATP in their high energy phosphate metabolic processes. For example, *E. histolytica* possesses a pyrophosphatedependent phosphofructokinase (11), a pyruvate-phosphate dikinase (12), a phosphoenolpyruvate carboxytransphosphorylase (13), and a pyrophosphate-acetate kinase (14). Several of these pyrophosphate-utilizing enzymes have also been discovered in other parasitic protists (see, *e.g.*, the discussion in Ref. 15), and more recently a pyruvate-phosphate dikinase (16) and a plantlike vacuolar proton-translocating pyrophosphatase (17–19) were discovered in many trypanosomatids as well.

An unusual characteristic of trypanosomatids in comparison with mammalian cells is the storage of calcium in acidic organelles which we termed acidocalcisomes (20). Initially identified in intact or permeabilized cells (21–23), these organelles have been isolated (17–19, 24) and found to have a high density and a high content of phosphorus, calcium, magnesium, sodium, and zinc (18, 19, 24). Our previous findings of large amounts of inorganic pyrophosphate in *T. cruzi* (10) are extended here to report the essentially complete ^{31}P NMR spectroscopic assignments of extracts of *T. cruzi* epimastigotes and their acidocalcisomes, together with the first reports of the ^{31}P NMR spectra and detailed assignments of whole cell and acidocalcisomal perchloric acid extracts of *T. brucei* and *Leishmania major*.

EXPERIMENTAL PROCEDURES

Culture Methods—T. brucei procyclic forms (ILTar 1 procyclics) and *L. major* promastigotes (WR-205 strain) were grown at 28 °C in medium SDM-79 (25) supplemented with 10% heat-inactivated fetal calf serum. *T. brucei* bloodstream forms (monomorphic strain 427 from clone MITat 1.4, otherwise known as variant 117) were isolated from infected mice or rats as described previously (26). *T. cruzi* Y strain epimastigotes were maintained at 28 °C in liver infusion tryptose medium (27) supplemented with 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml hemin. Protein was measured using the Bio-Rad Coomassie Blue method.

Chemicals—Phosphorylethanolamine, phosphorylcholine, sodium pyrophosphate, sodium tripolyphosphate, sodium tetrapolyphosphate,

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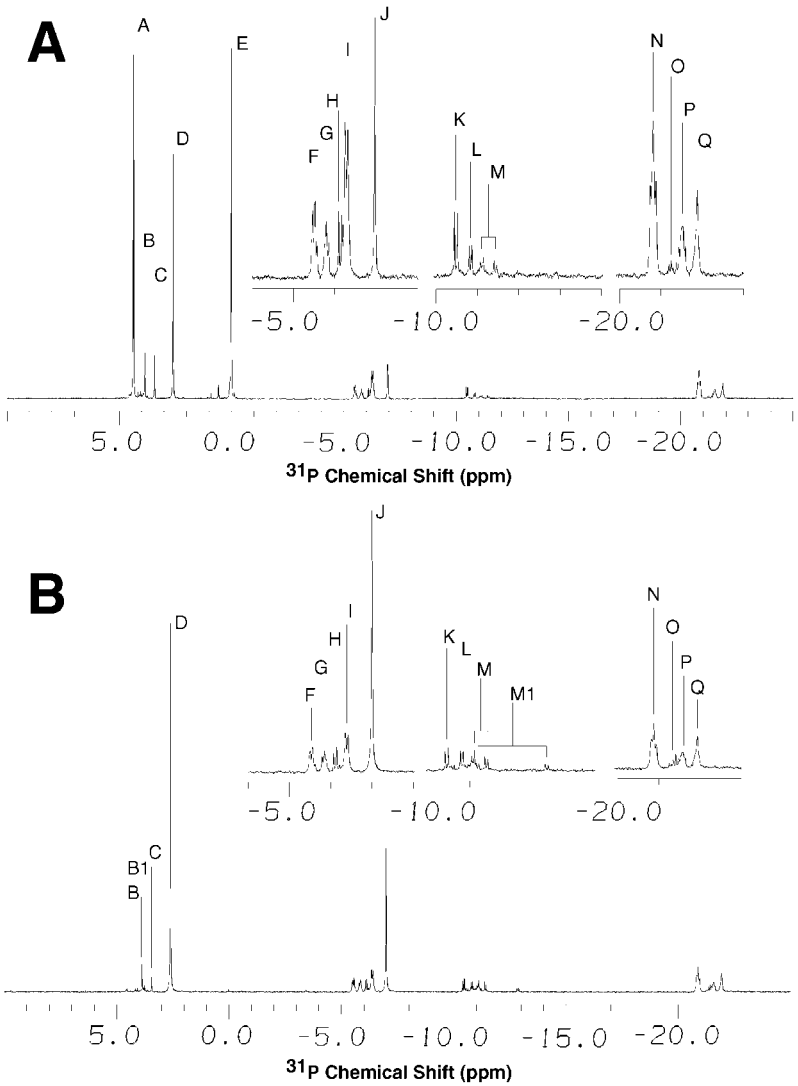

FIG. 1. **303.6-MHz (^{1}H decoupled) ^{31}P NMR spectra of perchloric acid extracts of *T. brucei* bloodstream forms (*A*) and *T. brucei* procyclic forms (*B*).** Both samples were pH 8, 20 mM EDTA, and 10% D_2O. The spectra were the accumulation of 1024 free induction decays acquired at room temperature with a 23-μs (90°) pulse excitation, recycle delay of 8 s (*A*) or 10 s (*B*), 20-kHz spectral width, and 16,384 data points. The FIDs were zero-filled once and apodized with 3-Hz exponential line broadening prior to Fourier transformation. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm (external reference). The *insets* show expansions of the upfield (−4 to −23 ppm) region of each spectrum, and essentially complete resonance assignments are given in Table I.

sodium trimetaphosphate, phosphate glass, *sn*-glycero-3-phosphate, glycerol-3-phosphorylcholine, ATP, ADP, AMP, phosphoarginine, dithiothreitol, leupeptin, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, uridine 5′-diphosphoglucose (UDPG), and NADH were from Sigma. 4-(2-Aminoethyl)benzenesulfonyl fluoride was from Calbiochem (San Diego, CA). Silicon carbide (400 mesh) was from Aldrich. Pepstatin was from Roche Molecular Biochemicals. Iodixanol (40% solution (OptiPrep), Nycomed) was obtained from Life Technologies, Inc. Coomassie Blue protein assay reagent was from Bio-Rad. All other reagents were analytical grade.

Isolation of Acidocalcisomes—Cells were collected by centrifugation and washed twice in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 50 mM Hepes, pH 7.2) and once in lysis buffer (125 mM sucrose, 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 20 mM K-Hepes, 5 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μM pepstatin, 10 μM leupeptin, 10 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, and 10 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, pH 7.2). The cell pellet was mixed with 1.5× wet weight silicon carbide and lysed by grinding in a pestle and mortar for at least 60 s. Lysis was monitored by optical microscopy. The lysate was clarified first by centrifugation at 144 × *g* for 5 min, then at 325 × *g* for 10 min. The second pellet was washed under the same conditions, and the supernatant fractions combined and centrifuged for 30 min at 10,500 × *g*. The pellet was resuspended in 4 ml of lysis buffer with the aid of a 22-gauge needle and applied to a discontinuous gradient of iodixanol, with 4-ml steps of 24%, 28%, 34%, 37%, and 40% iodixanol diluted in lysis buffer (28). The gradient was centrifuged at 50,000 × *g* in a Beckman SW 28 rotor for 60 min. The acidocalcisome fraction pelleted on the bottom of the tube and was resuspended in lysis buffer.

Perchloric Acid Extracts—For NMR, parasites (~ 1.5 g wet weight) were washed twice with buffer A containing 5.5 mM glucose, then extracted with ice-cold 0.5 M $HClO_4$ (2 ml/g wet weight cells). After 30 min of incubation on ice, the extracts were centrifuged at 3,000 × *g* for 5 min, and the supernatants neutralized by the addition of 0.72 M KOH, 0.6 M $KHCO_3$ (10). Precipitated $KClO_4$ was removed by centrifugation at 3,000 × *g* for 5 min, the supernatant separated and EDTA added to a final concentration of 20 mM prior to adjusting to pH 8. All extracts contained 10% D_2O (v/v) to provide a field-frequency lock.

Inorganic Pyrophosphatase Treatment—For the identification of pyrophosphate, extracts were incubated at 30 °C for 30 min (pH 7.2) with 10 units/ml of yeast inorganic pyrophosphatase (Sigma).

NMR Spectroscopy—Phosphorus NMR spectra were acquired at 303.6 MHz using a Varian INOVA NMR spectrometer equipped with a 17.6-tesla Oxford Instruments magnet. For perchloric acid extracts, 1024 or 4096 transients were typically collected at room temperature using a 23-μs (90°) pulse excitation, 20-kHz spectral width, 32,768 data points, and an 8- or 10-s recycle time. In most cases, proton decoupling was applied only during data acquisition to remove nuclear Overhauser effects and *J*-couplings. However, in some cases, proton-coupled spectra were used for the identification of phosphomonoester resonances. The connectivities between the α and β phosphate resonances in some acidocalcisomal samples were verified by phosphorus homodecoupling experiments with the decoupler frequency set at −20.8 ppm. The specific assignments of individual resonances were initially based on published chemical shifts (29) and ^{1}H-^{31}P and ^{31}P-^{31}P scalar couplings, and were then confirmed by co-addition of authentic reference compounds. The chemical shifts of all ^{31}P spectra were referenced to an 85% phosphoric acid external reference at 0 ppm (10), using the convention that high frequency, low field, paramagnetic, or deshielded values are positive (IUPAC δ-scale). Processing of NMR spectra and the calculation of relative intensities of the various ^{31}P resonances were performed using

TABLE I

^{31}P NMR resonance assignments for perchloric acid extracts (pH 8, 20 mM EDTA) of T. brucei bloodstream forms, T. brucei procyclics, T. cruzi epimastigotes, and L. major promastigotes

Peak	Assignment	Chemical shift (δ, ppm) and relative intensity (% total integral)							
		T. brucei bloodstream		*T. brucei* procyclics		*T. cruzi* epimastigotes		*L. major* promastigotes	
		δ	% total	δ	% total	δ	% total	δ	% total
A	Glycerol 3-phosphate	4.36	24.60						
A1	Glucose 6-phosphate					4.42	3.35	4.41	2.77
A2	3-Phosphoglycerate					4.05	2.32	4.21	2.31
B	Phosphorylethanolamine	3.85	2.57	3.87	2.25	3.86	3.82	3.90	3.04
B1	AMP			3.77	0.81	3.72	0.36	3.80	0.62
C	Phosphorylcholine	3.42	2.53	3.45	0.95	3.39	0.92	3.47	6.69
D	Inorganic phosphate	2.59	13.99	2.60	27.35	2.65	10.23	2.64	9.72
E	*sn*-Glycerophosphorylcholine	0.03	20.08					0.06	0.73
E1	*sn*-Glycerophosphorylethanolamine					−0.62	0.28	−0.49	0.15
E2	Phosphoarginine							−3.52	1.42
F	α-P of pentapolyphosphate	−5.52	3.00	−5.53	4.60	−5.52	5.03	−5.65	3.63
G	α-P of tetrapolyphosphate	−5.80	2.10	−5.86	4.24	−5.74	6.40	−5.90	2.71
H	β-P of ADP	−6.13	1.15	−6.01	2.11	−5.93	1.21		
I	α-P of tripolyphosphate	−6.25	7.05	−6.38	8.33	−6.23	15.34	−6.31	26.51
J	Pyrophosphate	−6.97	3.73	−7.00	18.48	−6.95	14.53	−7.00	11.62
K	α-P of ADP	−10.48	1.37	−10.42	1.71	−10.60	1.04	−10.50	0.87
L	α-P of ATP	−10.83	0.67	−10.81	1.58	−10.97	2.91	−10.86	0.45
M	NAD(H)+	−11.27	1.44	−11.20	2.23	−11.22	1.62	−11.21	1.54
M1	UDPG			−12.81	1.68	−12.73	2.46		
N	β-Ps of tri-, tetra-, and pentapolyphosphate	−20.82	8.62	−20.86	12.98	−20.89	20.19	−21.04	19.45
O	β-P of ATP	−21.26	0.46	−21.36	1.63	−21.33	1.31	−21.42	0.70
P	γ-P (central) of pentapolyphosphate	−21.54	1.58	−21.60	2.41	−21.58	2.56	−21.73	1.82
Q	Tetra-/pentametaphosphate	−21.86	3.21	−21.94	5.88	−21.98	2.20	−22.01	1.59

the FELIX 98 software package (Molecular Simulations Inc., San Diego, CA) running on a Silicon Graphics (SGI, Mountain View, CA) O2 workstation, and typically included base-line correction and 3-Hz exponential line broadening prior to Fourier transformation. Overall condensed inorganic phosphate levels were estimated from the NMR spectra by comparison with the α-ATP resonance (peak L, −10.8 ppm). A T_1 determination by the inversion recovery method (30) indicated that the condensed inorganic phosphates and nucleotide phosphates were fully relaxed under the experimental conditions used.

RESULTS

We show in Fig. 1 the 303.6-MHz ^{31}P (^{1}H decoupled) NMR spectra of perchloric acid extracts of *T. brucei* bloodstream (Fig. 1*A*) and procyclic (Fig. 1*B*) forms. The resonance assignments and relative intensities are given in Table I. The two most prominent resonances in the bloodstream form spectrum (*A* and *E*) occur at 4.36 and 0.03 ppm and are assigned to *sn*-glycerol-3-phosphate (G3P)[1] and glycerol-3-phosphoryl choline (GPC) based on their chemical shifts (29) and the results of co-addition of authentic G3P and GPC (data not shown). The presence of large amounts of G3P can be attributed to the well known specialized metabolism of the long slender form under anaerobic conditions (31). During centrifugation of the parasites, a decrease in the oxygen tension could lead to inactivation of the terminal mitochondrial oxidase (32) and the accumulation of G3P. This high concentration of G3P allows the parasite to maintain net production of ATP in the absence of oxygen via a reversible glycosomal glycerol kinase (32). The large amount of GPC observed could be the result of the process by which the long slender form acquires fatty acids from the host for the synthesis of membrane phospholipids (33). GPC has been observed to be the product of phospholipase A_1, an enzyme found in high concentrations in the plasma membranes of the parasite (33). Phospholipase A_1 cleaves the fatty acid chain of lysophosphatidylcholine, leaving free fatty acid and GPC as products (33). This process has been observed using ^{14}C labeling of lysophosphatidylcholine (34). In this previous study, GPC was detected in the extracellular medium, with no significant accumulation in the parasites (34), whereas in the bloodstream form extract shown here, it is clear that large amounts of GPC can accumulate within the cells. High levels of phosphorylethanolamine (*B*, 3.85 ppm) and phosphorylcholine (*C*, 3.42 ppm) are also present, consistent with the high levels of phosphatidylcholine and phosphatidylethanolamine in the glycosomal and plasma membranes (35).

The *insets* in Fig. 1 are expansions of the upfield (−4 to −23 ppm) regions of the ^{31}P NMR spectra, and both show major resonances in addition to the typical array of nucleotide di- and triphosphates, which are found in most cells. Sharp resonances corresponding to α-ADP (*K*), α-ATP (*L*), and NADH (*M*) are clearly visible in the −11 ppm region, and β-ADP (*H*) appears at −6.13 ppm. The β and γ phosphates of ATP resonate at −21.26 and −5.81 ppm (from direct spiking results), but overlap with additional peaks. The remaining resonances in the −5 to −7, and −20 to −22 ppm regions exhibit chemical shifts and spin-spin coupling patterns consistent with short chain condensed polyphosphates (36–39). Proceeding upfield, resonances *F* (−5.52 ppm, multiplet), *G* (−5.79 ppm, multiplet), *I* (−6.25, doublet), and *J* (−6.97, singlet) are assigned to the a (terminal) phosphates of (linear) penta-, tetra-, tri-, and pyrophosphate, respectively. The cluster of resonances at −20.82 ppm (*N*) can then be assigned to the β (bridging) phosphorus atoms of the tri- and tetrapolyphosphate, as well as phosphates 2 and 4 of pentapolyphosphate. The resonance appearing at −21.27 ppm is assigned to the centermost bridging phosphorus atom of pentapolyphosphate, based on previous observations of pentapolyphosphate (36, 39), and supported by the relative intensity of this resonance compared with the −5.52 ppm resonance cluster. To confirm these assignments, authentic pyro-, tri-, and tetrapolyphosphate were added to samples and found to co-resonate with the peaks in question, with the β phosphorus of tripolyphosphate and the β phosphorus of tetrapolyphosphate comprising the downfield and upfield components of the

[1] The abbreviations used are: G3P, *sn*-glycerol-3-phosphate; GPC, glycerol-3-phosphoryl choline; UDPG, uridine 5′-diphosphoglucose.

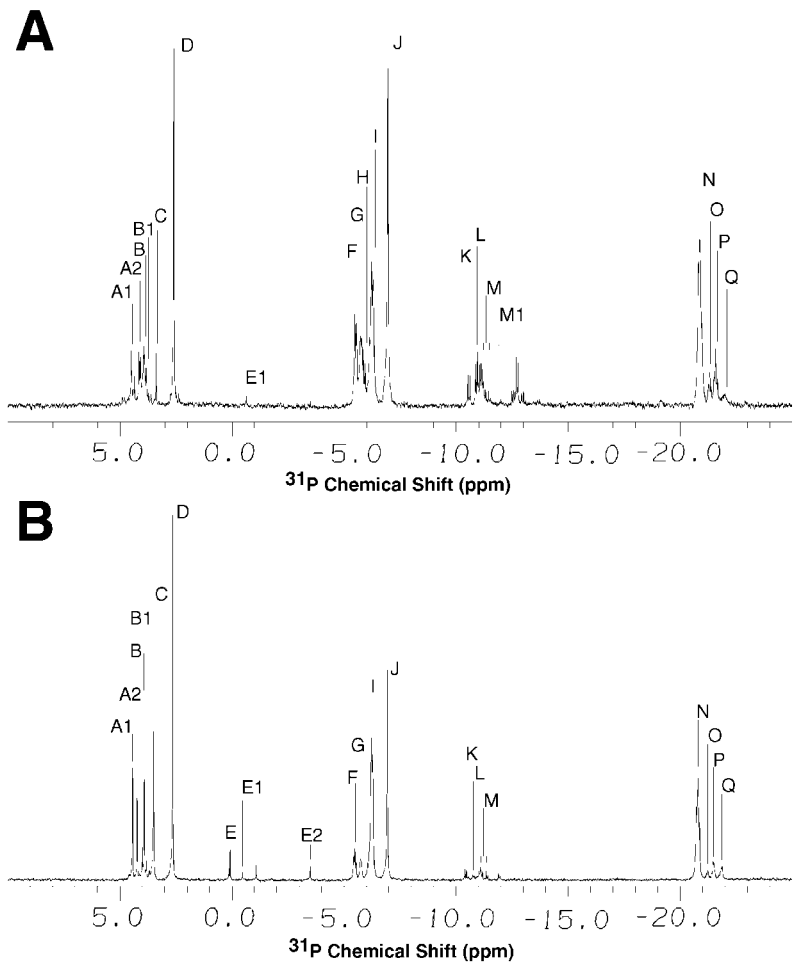

FIG. 2. **303.6-MHz (^{1}H decoupled) ^{31}P NMR spectra of perchloric acid extracts of *T. cruzi* epimastigotes (*A*) and *L. major* promastigotes (*B*).** Both samples were pH 8, 20 mM EDTA, and 10% D_2O. The spectra were the accumulation of 1024 free induction decays acquired at room temperature with a 23-μs (90°) pulse excitation, 8-s recycle delay, 20-kHz spectral width, and 16,384 data points. The FIDs were zero-filled once and apodized with 3-Hz exponential line broadening prior to Fourier transformation. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm (external reference). Resonance assignments are given in Table I.

−20.82 ppm resonance, respectively. Finally, the peak at −21.62 ppm, lacking any significant multiplicity, is tentatively assigned to cyclic tetrametaphosphate and/or pentametaphosphate (the latter resonating upfield by 0.1 ppm), based on previous reports of these species in the literature (37). Interestingly, these extracts show no significant (cyclic) trimetaphosphate (−21.40 ppm), the primary breakdown product of longer chain polyphosphates by chemical degradation, indicating that all these inorganic phosphates are unlikely to be the result of very long chain polyphosphate hydrolysis during extract preparation (40, 41). Similarly, we found no evidence for these short chain polyphosphates when a sample of long chain polyphosphate was subjected to our perchloric acid extraction protocol (data not shown). There are, therefore, high levels of condensed short chain polyphosphates in acid extracts of *T. brucei*, and by inference, in the natural state of *T. brucei* parasites themselves.

Fig. 2 shows the 303.6-MHz ^{1}H-decoupled ^{31}P NMR spectra of perchloric acid extracts of the insect forms of two other trypanosomatid parasites: *T. cruzi* and *L. major*, and resonance assignments and relative intensities are given in Table I. The phosphomonoester (5 to 0 ppm) regions of the ^{31}P NMR spectra of the perchloric acid extracts of these parasites are qualitatively similar to these seen with the *T. brucei* procyclic forms, with the detection of the metabolic intermediates G6P and 3-PGA, together with phosphorylcholine (particularly elevated in the case of *L. major*) and phosphorylethanolamine. The phosphagen phosphoarginine, responsible for ATP homeostasis during muscle contraction in some invertebrates (42–44), was detected at −3.52 ppm in *L. major* at approximately half the level of ATP, and at trace levels in *T. cruzi*. A phosphoarginine kinase with a putative actinin-like actin binding site has recently been cloned and characterized in *T. cruzi*, and is hypothesized to be involved in cytoskeletal movements requiring high energy consumption (45). In the upfield region (from ~−5 ppm), there are once again strong resonances corresponding to short chain polyphosphates, with additional peaks arising from ADP, ATP, NAD(H), and UDPG. In both *T. cruzi* and *L. major*, however, the vast majority of the observed spectral intensity arises from condensed phosphates, primarily pyrophosphate and tripolyphosphate, just as found with the *T. brucei* bloodstream and procyclic spectra shown in Fig. 1.

By comparison with published ATP levels (10, 31, 46–48), the overall condensed inorganic phosphate levels in the trypanosomes can be estimated to be ~ 200 nmol/mg of protein, on average. In *T. cruzi* epimastigotes, the level measured here by NMR is ~ 260 nmol/mg of protein, some 20% higher than the enzymatically determined pyro- and tripolyphosphate level of 195 nmol/mg of protein reported previously (10). However, this previous measurement did not include contributions from tetra- or pentapolyphosphate, and scaling the present value down to reflect only the pyro- and tripolyphosphate levels gives 188 nmol/mg of protein, very good agreement. *L. major* promastigotes contain the highest level of condensed inorganic phosphates, ~ 350 nmol/mg of protein, while both forms of *T. brucei* contain ~100–200 nmol/mg of protein, values that are related to ATP levels (31) and therefore depend on the oxygen tension during sample preparation, which was not controlled in this study.

The subcellular localization of these abundant polyphosphates is expected to be the acidocalcisome, a novel calcium, magnesium, sodium, zinc, and phosphorus storage organelle

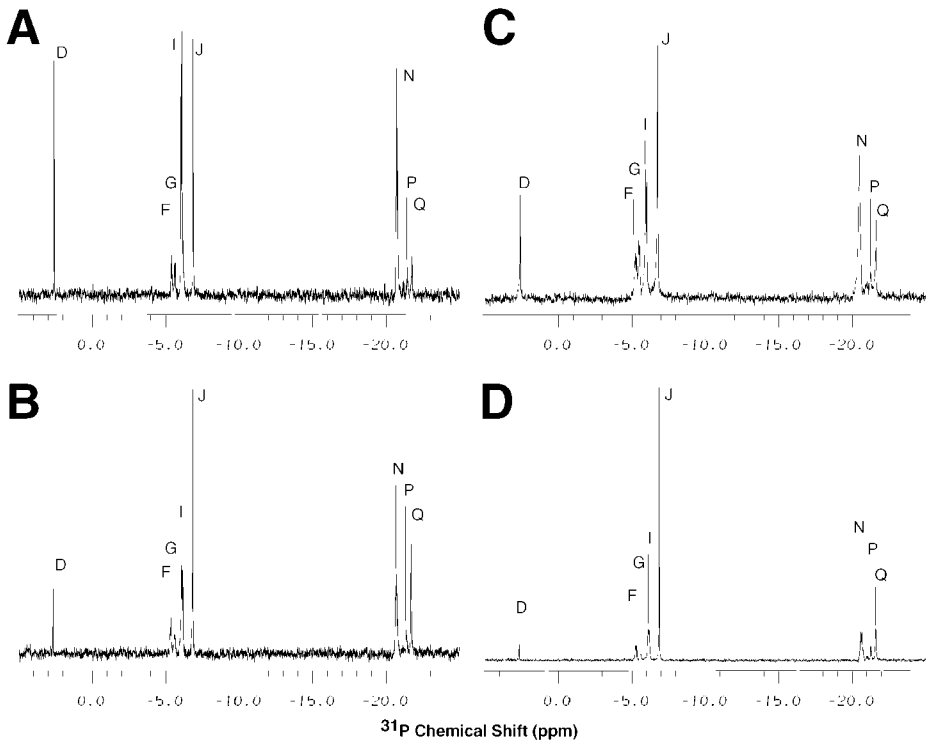

FIG. 3. **303.6-MHz (^{1}H decoupled) ^{31}P NMR spectra of perchloric acid extracts of isolated acidocalcisomes.** Figure shows *T. brucei* bloodstream forms (*A*), *T. brucei* procyclics (*B*), *T. cruzi* epimastigotes (*C*), and *L. major* promastigotes (D), all at pH 8, 20 mM EDTA, and 10% D_2O. The spectra were the accumulation of 4096 (*A*) or 2048 (*B–D*) free induction decays acquired at room temperature with a 23-μs (90°) pulse excitation, recycle delay of 8 s (*A*) or 10 s (*B–D*), 20-kHz spectral width, and 16,384 data points. The FIDs were zero-filled once and apodized with 3-Hz exponential line broadening prior to Fourier transformation. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm (external reference). Resonance assignments are given in Table II.

TABLE II

^{31}P NMR resonance assignment for perchloric acid extracts (pH 8, 20 mM EDTA) of acidocalciosmes from T. brucei bloodstream forms, T. brucei procyclics, T. cruzi epimastigotes, and L. major promastigotes

Peak	Assignment	Chemical shift (ppm) and relative intensity (% total integral)							
		T. brucei bloodstream		*T. brucei* procyclics		*T. cruzi* epimastigotes		*L. major* promastigotes	
		δ	% total	δ	% total	δ	% total	δ	% total
D	Inorganic phosphate	2.60	7.33	2.67	4.57	2.59	5.53	2.61	2.8
F	α-P of pentapolyphosphate	−5.36	5.49	−5.30	6.94	−5.29	6.99	−5.36	7.12
G	α-P of tetrapolyphosphate	−5.60	4.60	−5.60	4.74	−5.51	8.63	−5.65	2.18
I	α-P of tripolyphosphate	−6.08	35.05	−6.11	23.17	−6.04	17.60	−6.22	16.70
J	Pyrophosphate	−6.87	10.05	−6.85	20.79	−6.76	17.91	−6.92	36.44
N	β-Ps of tri-, tetra-, and pentapolyphosphate	−20.73	28.31	−20.69	21.67	−20.47	24.45	−20.67	17.49
P	γ-P (central) of pentapolyphosphate	−21.46	2.90	−21.38	3.70	−21.25	3.43	−21.36	3.89
Q	Tetra-/pentametaphosphate	−21.78	4.58	−21.71	14.44	−21.62	10.69	−21.67	12.20

common to all trypanosomatids investigated to date (20). To test this idea in more detail, acidocalcisomes were isolated from *T. brucei*, *T. cruzi*, and *L. major* using an iodixanol (OptiPrep) density gradient (28). The densest fraction, corresponding to the acidocalcisomes, was then extracted with perchloric acid, treated with 20 mM EDTA to chelate the divalent metal cations, and observed by high resolution ^{31}P NMR (Fig. 3, *A–D*). The spectra of the acidocalcisomal contents of all three species are very similar and resemble those of the whole cell extracts (Figs. 1 and 2), but contain only the short chain inorganic polyphosphate resonances, corresponding to pyro-, tri-, tetra-, and pentapolyphosphate, as well as tetra- and/or pentametaphosphate. In previous work with *T. cruzi* (24), x-ray microprobe analysis revealed high levels of calcium (171 ± 6), magnesium (646 ± 19), sodium (161 ± 18), zinc (148 ± 6) and phosphorus (1390 ± 13 mmol/kg dry weight) in acidocalcisomes, and from these results and the requirement of overall charge neutrality, a charge-per-phosphorus ratio of ~1.6 was estimated (24). This x-ray measurement translates to an average chain length of 3.33 phosphates/anion, which is very close to the values that we obtain here from the integrated intensities of the α and β phosphate groups. In acidocalcisomes from *T. brucei* bloodstream forms, *T. brucei* procyclics, *T. cruzi* epimastigotes, and *L. major* promastigotes, the average chain lengths are 3.11, 3.39, 3.25, and 3.12, respectively. There is therefore good agreement between the average phosphate chain lengths, as determined by x-ray microprobe analysis and NMR spectroscopy, although the NMR experiments have the advantage of a more direct and accurate determination of the precise chain length distribution.

DISCUSSION

The presence of polyphosphates in various microorganisms is well established and the hypothetical roles of these molecules have been reviewed (40, 41, 49). The localization of these molecules within the cation-rich acidocalcisomes implies that their

functional roles could be 1) energy stores and/or 2) chelators of metal ions. For example, in plants and in some parasitic protozoa, pyrophosphate is used in place of ATP as an energy donor in several reactions (reviewed in Ref. 15), such as the pyruvate, phosphate dikinase of trypanosomatids (16). The vacuolar-type proton-translocating pyrophosphatase, which has now been located in the acidocalcisomal membranes of *T. cruzi* (17), *L. donovani* (18), *T. brucei* (19), and *T. gondii* (50), is also of interest, having been previously described only in plants. Moreover, this enzyme has also recently been identified in malaria parasites (51, 52). The parasite's V-H^+-PPase has been shown to drive proton uptake into the acidocalcisomal compartment through cleavage of cytosolic pyrophosphate, and this activity is inhibited by small pyrophosphate analogs, such as imidodiphosphate, in a similar manner to that seen in plant systems (53). Indeed, the sequences of the V-H^+-PPases in *T. cruzi* (AF159881), *P. falciparum* (AF115766), and *Arabidopsis thaliana* (A6005697) bear an extremely high sequence homology to each other, and the V/H^+-PPase appears to be an attractive target for drug development.

Polyphosphates undoubtedly form metal complexes within the acidocalcisome and binding studies of pure short chain polyphosphates have been performed, which indicate that in most cases soluble 1:1 complexes are formed with both magnesium and calcium, with precipitation occurring at 2–3 mol of metal/mol of polyphosphate (54). Because of their high electron density, the acidocalcisomes appear as dark vacuoles in transmission electron micrographs of unstained cells. This density is released from the acidocalcisomes by light permeabilization with formaldehyde, followed by treatment with yeast inorganic pyrophosphatase (10), implying a close association between the short chain polyphosphates and the metals, as would be expected from the high concentrations of these ions in this organelle (~3 M). Previous NMR experiments using the same yeast inorganic pyrophosphatase for peak identification have shown that pyrophosphate, and to a lesser extent tripolyphosphate, are hydrolyzed by the enzyme to inorganic phosphate (10). Together with the above observation of electron density loss from the acidocalcisomes, this suggests that the polyphosphate structure plays a role in cation storage and transport.

Indeed, it is currently hypothesized that one of the main roles of the acidocalcisome in *T. cruzi* is calcium storage for use in intracellular signaling, particularly in the infective stages (20). Intracellular amastigotes survive in a low concentration of free calcium in the host cytosol (~0.1 mM compared with 1–2 mM extracellularly), and the acidocalcisome may form an internal releasable calcium reserve (20). As the polymer structure of the polyphosphates is required for efficient cation complexing, it is intriguing that a gene with homology to exopolyphosphatases has recently been found in *L. major* (55), providing the first evidence for a breakdown pathway for these molecules in trypanosomatids. Exopolyphosphatases have been found in prokaryotes and eukaryotes, and cleave orthophosphate from the end of the polyphosphate chain. Although in bacteria these enzymes hydrolyze mostly high molecular weight polyphosphates (56), at least some of the enzymes from yeast are more active at hydrolyzing short chain polyphosphates such as tripolyphosphate (56). Endopolyphosphatases that act on long chain polyphosphates generating tripolyphosphate have also been detected in eukaryotes, including the protozoan *Giardia lamblia* (57). Interestingly, the yeast endopolyphosphatase is localized within vacuoles (57). Other enzymes that cleave tripolyphosphate have also been reported in different organisms (58).

Similar enzymes may operate under certain conditions in acidocalcisomes to cleave tripoly- (and possibly longer) phosphate to pyrophosphate and orthophosphate, as a means of regulating calcium content. Additionally, the pyro- and orthophosphate may also be exported to the cytosol where they can be utilized by pyrophosphatases, kinases, etc. Here, it may be noteworthy that the acidocalcisomal polyphosphates would only be accessible to polyphosphatases on the inside of the acidocalcisome, while the V-H^+-PPase only pumps protons when driven by pyrophosphate on the outside of the acidocalcisome.

In conclusion, the results shown above indicate the presence of large quantities of short chain polyphosphates in *T. cruzi*, *T. brucei*, and *L. major*, the causative agents of Chagas' disease, African sleeping sickness, and leishmaniasis. Given the identification of at least two pyrophosphate-utilizing enzymes in each organism, including the ubiquitous plantlike V-H^+-PPase, it appears that short chain polyphosphates could play an important role in parasite metabolism, and that plantlike pyrophosphatases (and potentially tripolyphosphatases) may be future candidates for chemotherapy.

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