Sugar Nucleotide Pools of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*††

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The cell surface glycoconjugates of trypanosomatid parasites are intimately involved in parasite survival, infectivity, and virulence in their insect vectors and mammalian hosts. Although there is a considerable body of work describing their structure, biosynthesis, and function, little is known about the sugar nucleotide pools that fuel their biosynthesis. In order to identify and quantify parasite sugar nucleotides, we developed an analytical method based on liquid chromatography-electrospray ionization-tandem mass spectrometry using multiple reaction monitoring. This method was applied to the bloodstream and procyclic forms of *Trypanosoma brucei*, the epimastigote form of *T. cruzi*, and the promastigote form of *Leishmania major*. Five sugar nucleotides, GDP-α-Man, UDP-α-Man-N-acetylglucosamine, UDP-α-Glc-galactopyranosyl-α-Man and GDP-β-L-Fuc, were common to all three species; one, UDP-α-D-galactofuranosyl, was common to *T. cruzi* and *L. major*; three, UDP-β-D-rhamnopyranosyl, UDP-α-D-xylose, and UDP-α-D-glucuronic acid, were found only in *T. cruzi*; and one, GDP-α-D-arabinopyranosyl, was found only in *L. major*. The estimated demands for each monosaccharide suggest that sugar nucleotide pools are turned over at very different rates, from seconds to hours. The sugar nucleotide survey, together with a review of the literature, was used to define the routes to these important metabolites and to annotate relevant genes in the trypanosomatid genomes.

The trypanosomatids are protozoan parasites that impose a major health burden on many countries in the developing world, causing a wide range of diseases over three continents. Like most eukaryotes, the cell surfaces and endosomal/lysosomal systems of these organisms are rich in glycoconjugates, some of which play essential roles in their survival, infectivity, or virulence. The glycoconjugate repertoires of the three trypanosomatids studied here, *Trypanosoma brucei*, *T. cruzi*, and *Leishmania major*, are fundamentally different, reflecting their disparate life cycles, modes of infection, and disease pathologies (see references 5, 27, 28, 38, 47, 65, and 69 and references therein). The monosaccharides that make up these glycoconjugates vary between the three trypanosomatid species. For example, all three contain D-mannose (Man), D-N-acetylglucosamine (GlcNAc), D-glucosamine (GlcN), D-glucose (Glc), and D-galactopyranosyl (Galp), while only *T. cruzi* and *L. major* contain D-galactofuranosyl (Galp), only *T. cruzi* contains D-xylose (Xyl), D-rhamnopyranosyl (Rha), and D-fucose (Fuc), and only *L. major* contains D-arabinopyranosyl (Ara) (Table 1). However, a unifying theme of their glycobiology is an abundance of cell surface glycosylphosphatidylinositol (GPI)-anchored glycoproteins and/or non-protein-linked GPI structures.

The protein-linked GPI glycan of the leishmania are the simplest in structure, consisting of the conserved Manα1-2Manα1-6Manα1-4GlcN core. Those of *T. cruzi* are principally Manα1-2Manα1-2Manα1-6Manα1-4GlcN, and those of *T. brucei* are the most complex, with α-D-Galp and β-D-Galp side chains in the bloodstream form and sialylated poly-N-acetylgalactosamine (poly-LacNAc) side chains in the procyclic form (27).

The non-protein-linked GPI structures include the so-called glycosylphosphatidylcholines, or GPCls, that are most abundant in the leishmania and *T. cruzi* species (58, 64, 90) but are also found in procyclic form *T. brucei* (60, 73, 122) and bloodstream form *T. cruzi* (39, 63). Non-protein-linked GPCls include the more exotic leishmania-specific lipophosphoglycan (LPG) structures (38, 47, 64, 65). The leishmania LPGs contain characteristic phosphosaccharide repeats of Galpβ1-4Manα1-1, which in *L. major* can have β-D-Galp and α-D-Arap side chains (66, 67). These repeats are also found in the secreted filamentous and GPI-anchored membrane proteophosphoglycans (PPGs) of *L. major* (47). The phosphosaccharide repeat chains of leishmania PPGs are attached to protein via Manα1-1-P-Ser linkages (49). Although considerably less abundant, P-linked glycans have also been found in *T. cruzi*, in gp72 (40) and the NETNES glycoprotein (41), and in *T. congolense* GARP (113). They may also exist in the *T. brucei* gp72 homologue Fla-1 (77). The occurrence of P-linked glycans in nature is highly restricted and, outside the trypanosomatids, has been reported as occurring only in *Dictyostelium* (108) and *Entamoeba* (70). Whereas the phosphosaccharides of NETNES are composed of mannose chains only, those of gp72 are extremely complex, containing α-L-Fuc, α-L-Rha, and β-D-Xyl as well as GlcNAc, Galp, and GalpF does not occur in mammals but is abundant in *T. cruzi* and the leishmania, where it occurs in the LPG cores and some GIPs. On the other hand, for the three trypanosomatids, Fuc, Rha, and Xyl have been found

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| Sugar | T. brucei bloodstream form occurrence of: | T. brucei procyclic form occurrence of: | T. cruzi epimastigote form occurrence of: | L. major promastigote form occurrence of: |
|-------|------------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| GDP-Man | + | - | + | - |
| GDP-GlcNAc | - | + | - | + |
| GDP-Glc | - | - | + | - |
| GDP-Ara | - | - | - | + |
| GDP-Fuc | - | - | - | + |
| GDP-NANA | - | - | - | + |
| UDP-Man | - | - | - | + |
| UDP-GlcNAc | - | - | - | + |
| UDP-Glc | - | - | - | + |
| UDP-Ara | - | - | - | + |
| UDP-Fuc | - | - | - | + |
| UDP-NANA | - | - | - | + |
| fucose | - | - | - | + |
| D-Man | + | - | + | - |
| D-Man | - | + | - | + |
| D-Man | - | - | + | - |
| D-Man | - | - | - | + |
| fucose | - | - | - | + |
| D-GlcNAc | + | - | + | - |
| D-GlcNAc | - | + | - | + |
| D-GlcNAc | - | - | + | - |
| D-GlcNAc | - | - | - | + |
| fucose | - | - | - | + |
| D-Galp | + | - | + | - |
| D-Galp | - | + | - | + |
| D-Galp | - | - | + | - |
| D-Galp | - | - | - | + |
| fucose | - | - | - | + |
| D-Xyl | + | - | + | - |
| D-Xyl | - | + | - | + |
| D-Xyl | - | - | + | - |
| D-Xyl | - | - | - | + |
| fucose | - | - | - | + |
| L-Rhap | + | - | + | - |
| L-Rhap | - | + | - | + |
| L-Rhap | - | - | + | - |
| L-Rhap | - | - | - | + |
| fucose | - | - | - | + |
| D-Arap | + | - | + | - |
| D-Arap | - | + | - | + |
| D-Arap | - | - | + | - |
| D-Arap | - | - | - | + |
| fucose | - | - | - | + |
| L-Fucp | + | - | + | - |
| L-Fucp | - | + | - | + |
| L-Fucp | - | - | + | - |
| L-Fucp | - | - | - | + |
| fucose | - | - | - | + |
| 2-3 sialosides* | + | - | + | - |
| 2-3 sialosides* | - | + | - | + |
| 2-3 sialosides* | - | - | + | - |
| 2-3 sialosides* | - | - | - | + |
| fucose | - | - | - | + |

* Known.

The presence (+) or absence (−) of a particular sugar in a given organism is indicated in the "All" column for that organism. The "GPI" column refers to the presence or absence of that sugar in any of the following specific structural categories, as indicated: protein GPI anchors, free GIPLs, protein N-linked glycans, protein O-linked glycans, protein phosphodiester-linked glycans (P-glycan), and base J. If a sugar is known or presumed to be present in a given organism, it is indicated in the "All" column for that organism. The "GPI" column refers to the presence or absence of that sugar in any of the following specific structural categories, as indicated: protein GPI anchors, free GIPLs, protein N-linked glycans, protein O-linked glycans, protein phosphodiester-linked glycans (P-glycan), and base J. If a sugar is known or presumed to be present in a given organism, it is indicated in the "All" column for that organism.
only in *T. cruzi*. *T. cruzi* is also unique in expressing abundant O-linked glycans with GlcNAc3-1-O-Thr linkages (89). These are found on all life cycle stages in GPI-anchored mucin-like molecules and contain mostly βGalp, which can be α2-3 sialylated by the action of cell surface trans-sialidases (1, 14, 88).

All three of the trypanosomatid express N-linked glycans. The dolichol (Dol) cycles of N-glycan precursor biosynthesis are atypical in trypanosomatids in that they lack the glucosyltransferases (Alg6, Alg8, and Alg10) to make the GlcMan3Nac-P-P-Dol precursor common to most other eukaryotes (99). The mature Dol-PP oligosaccharide species used for transfer to protein vary according to trypanosomatid species. For example, *L. mexicana* utilizes biantennary Man3GlcNAc2-PP-Dol, whereas *T. cruzi* utilizes Man3GlcNAc2-PP-Dol during most of its life cycle but uses both Man4GlcNAc2-PP-Dol and Man5GlcNAc2-PP-Dol in its bloodstream trypomastigote stage (82). Procylic-form *T. brucei* uses Man3GlcNAc2-PP-Dol (2), while bloodstream form *T. brucei* uses either Man4GlcNAc2-PP-Dol or Man5GlcNAc2-PP-Dol, depending on the glycosylation site (51). The final, fully processed, mature N-linked glycans of the leishmania are atypical biantennary oligomannose structures, some bearing an αGlcp residue (80), while those of *T. cruzi* tend to be conventional oligomannose- and complex-type structures (83), though some also carry Galβ1,9 while others are sulfated, and a small fraction contain Fucβ (8). The *T. brucei* structures are predominantly conventional oligomannose-type structures in the procyclic form (116) and a mixture of conventional oligomannose- and complex-type structures (125, 126) and unique giant poly-LacNAc-containing structures in the bloodstream form (5). The latter are located primarily in the flagellar pocket and endosomal/lysosomal system of the parasite.

The information above, summarized in Table 1, defines the glycosyl donor requirements of each organism. Based on precedent, it is likely that most of the glycosyl donors are conventional sugar nucleotides (e.g., GDP-mannose [GDP-Man], GDP-fucose [GDP-Fuc], UDP-N-acetylglucosamine [UDP-GlcNAc], UDP-glucose [UDP-Glc], UDP-galactopyranose [UDP-Galp], UDP-galactofuranose [UDP-Galf], and UDP-xyllose [UDP-Xyl]), while our previous work has shown that the donor of the relatively rare sugar Ara is GDP-α-D-Ara (GDP-Ara) (100). The identity of the 1-rhamnopyranose donor was unknown, but as a result of this study, we have identified it as UDP-rhamnose (UDP-Rha).

In this paper, we describe a new liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ES-MS/MS) method to identify and quantify sugar nucleotides in cell extracts and its application to three trypanosomatid parasites. The results are presented in the context of our knowledge about the glycobiology of these organisms and their recently completed genomes (11, 25, 50).

**MATERIALS AND METHODS**

**Chemicals and reagents.** Commercially available sugar nucleotide standards UDP-Galp, UDP-Glc, UDP-GlcNAc, GDP-Man, GDP-Fuc, and GDP-Glc were purchased from Sigma-Aldrich. UDP-Galp was a kind gift from Andrei Nikolaeff (University of Dundee), and GDP-Rha and UDP-Xyl were kind gifts of Ben Davies (University of Oxford). High-performance liquid chromatography (HPLC)-grade solvents were from VWR, and sequanal-grade triethylamine was from Pierce. High-grade (40%) sodium hydroxide was from BDH, and “Suprapur” sodium acetate was from Merck. A 200 mM stock solution of triethylammonium acetate (TEAA) buffer was made by adjusting the pH of triethylamine to pH 6 using “Aristar” acetic acid (BDH). The TEAA stock solution was frozen in aliquots at −20°C until use.

**Cell culture.** *T. brucei* bloodstream form parasites (strain 427, variant MITat 1.2) were cultured in HMI-9 medium with 10% fetal calf serum (FCS; PAA Laboratories) (43) at 37°C with 5% CO2. For extraction of sugar nucleotides, cells were harvested at ~1 x 10⁶/ml. Total cell volume was assumed to be 52 μl per 10⁶ cells (37). *T. brucei* procyclic-form parasites were cultured in SDM-79 medium with 10% FCS (13) at 28°C. For extraction of sugar nucleotides, cells were harvested at ~1 x 10⁶/ml. Total cell volume was assumed to be 48 μl per 10⁶ cells (21). *L. major* promastigotes (Friedlin strain) were grown in M-199 medium with 10% FCS (16) at 28°C in a shaking incubator. Cells were harvested at 5 x 10⁶/ml for sugar nucleotide analysis. Total cell volume was assumed to be 35 μl per 10⁶ cells (M. McConville, personal communication). *T. cruzi* epimastigotes (CL Brener strain) were grown in RTH medium (45, 86) supplemented with 10% FCS at 28°C and were harvested at 4 x 10⁶/ml for sugar nucleotide analysis. Total cell volume was assumed to be 55 μl per 10⁶ cells (59). Wild-type CHO (Chinese hamster ovary) cells (a kind gift of Paul Crocker) were grown in Ham’s F-12 medium plus 10% FCS. Cells were grown at 37°C, 5% CO2. Cells were harvested at approximately 50% confluence for sugar nucleotide analysis. Cell volume was estimated as 12 μl per 10⁶ cells (46).

**Sugar nucleotide extraction.** Cells were pelleted by centrifugation, washed once in ice-cold phosphate-buffered saline, and then lysed with cold (~20°C) 70% ethanol on ice in the presence of a 20 pmol GDP-glucose internal standard (Sigma). The typical numbers of cells per analysis were 1 x 10⁶ for bloodstream-form *T. brucei, 5 x 10⁶ for procyclic-form *T. brucei, and promastigote form*. The lysate was centrifuged at 16,000 x g for 10 min at 4°C to remove insoluble material, and the supernatant was dried under nitrogen. The samples were dissolved in 200 μl of 9% butan-1-ol and extracted three times with 400 μl of 90% butanol to remove lipids. The resulting aqueous phase was dried under nitrogen and resuspended in 5 mM ammonium bicarbonate, and sugar nucleotides were extracted using EmpCarb graphitized carbon columns (Supelco) as previously described (92). Briefly, columns were prepared by washing with 3 ml of 80% acetonitrile, 0.1% trifluoroacetic acid followed by 2 ml water. The sample was loaded in 5 ml ammonium bicarbonate, and the column was washed with 2 ml water, 2 ml of 25% acetonitrile, and 2 ml 50 mM TEAA buffer and eluted with 2 ml of 25% acetonitrile, 50 mM TEAA buffer. The eluate was freeze-dried and stored at −80°C prior to analysis.

**Reverse-phase HPLC of sugar nucleotides.** HPLC conditions were adapted from reference 92. A 1- by 250-mm C18 column (HiChrom) was used. The initial conditions were 0.5% acetonitrile, 20 mM TEAA buffer (pH 6) for 15 min at a flow rate of 20 μl/min followed by a linear gradient from 0.5 to 4% acetonitrile in 20 mM TEAA buffer over 20 min at a flow rate of 25 μl/min. The column was then washed for 20 min in 4% acetonitrile, 20 mM TEAA buffer before reequilibration into 0.5% acetonitrile, 20 mM TEAA buffer before running the next sample. 

**ES-MS of sugar nucleotides.** ES-MS was performed on a Quattro Ultima triple quadrupole mass spectrometer (Waters) in negative ion mode using a slow-flow source with a capillary voltage of 3 kV and a cone voltage of 70 V. To establish mass transitions for sugar nucleotide standards, samples were introduced at 10 μl per minute using a Harvard syringe pump. Spectra were collected, analyzed, and processed using MassLynx software (Waters). For ES-MS/MS product ion scanning, the argon collision gas pressure was set to 3 x 10⁻⁵ Torr and the collision voltage was 28 V. For LC-MS/MS runs, the flow from the HPLC was made up to 50 μl/min with acetonitrile delivered to a mixing T piece by a Harvard syringe pump. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode for a number of predetermined mass transitions. To accommodate the higher flow rate, the source temperature was raised to 110°C and the desolvation temperature set to 375°C. Chromatograms were recorded for 40 min, and peak areas were integrated automatically using the MassLynx software.

**HPLC of sugar nucleotides.** Sugar nucleotides were separated by high-pH anion-exchange chromatography (HPAEC) using a Dionex LC system and Chromelon software. HPLC conditions were based upon reference 115, and a 2- by 250-mm CarboPac PA1 column (Dionex) was used at a flow rate of 0.25 ml/min. Sugar nucleotides were detected by UV absorbance at 260 nm. The chromatograms were used to estimate the purity of standard solutions and to determine the relative ratios of sugar nucleotide standards in standard mixtures used to calibrate the LC-MS/MS system.
Measurement of sugar nucleotides in trypanosomatid parasites. There is evidence that sugar nucleotide concentration can change with the growth phase of cells (55). Consequently, only cells that were in the mid- to late log phase were used for our analyses. For each cell type, at least four different cell suspensions were analyzed by negative ion ES-MS to find the \([M-H]^-\) ion at \(m/z\) 442 corresponding to a \([GDP-H]^+\) product ion. For example, GDP-Fuc is observed as the \([M-H]^-\) ion at \(m/z\) 588 in the ES-MS spectrum that, when fragmented, produces a major product ion at \(m/z\) 442 corresponding to a \([GDP-H]^+\) ion (see Fig. S1 in the supplemental material). This defines the MRM transition for GDP-Fuc as \(m/z\) 588 to \(m/z\) 442. The MRM transitions for the sugar nucleotides analyzed in this work are shown in Table 2. In all cases, the major product ion at \(m/z\) 442 gives rise to a specific fragment ion. Thus, only when a molecular ion gives rise to a specific fragment ion can the ion recorded and assigned.

In order to make our analyses quantitative, we added a known amount of an internal standard (GDP-Glc, a sugar nucleotide that is not present in trypanosomatid sugar nucleotide extracts) at the point of cell extraction. The molar relative response factors (MRRFs) relative to this internal standard can vary slightly from day to day due to ion source and collision cell conditions. Therefore, we determined the MRRF values for each batch of analyses by running a mixture of commercially available sugar nucleotides (see Fig. S2A in the supplemental material). Sugar nucleotide levels in cell extracts were then calculated using the following formula: quantity of \(x\) pmol \(= x/\text{peak area of GDP-Glc} \times \text{pmoles of GDP-Glc added to extract/MRRF}\).

The measurement of sugar nucleotides in CHO mammalian cell line and comparing our results with those previously obtained by HPAEC and UV detection (115) (see Table S1 in the supplemental material). Our values are very similar to those previously reported, with the exceptions of our identification of GDP-Man, which has not been previously detected in CHO cell sugar nucleotide analyses, and of the figure for GDP-Man, where our measurement is fourfold lower that that previously obtained. The measurement of GDP-Man discrepancy is unclear but may be due to confounding UV-absorbing material coeluting with this low-abundance sugar nucleotide in the previously published HPAEC system.

**RESULTS**

Establishing an LC-MS/MS method for the identification and quantification of sugar nucleotides. In order to sensitively and quantitatively survey the sugar nucleotide pools of trypanosomatid parasites, we devised an appropriate extraction and analysis protocol. The criteria were that the method should be (i) sensitive and quantitative with a wide dynamic range (to allow the simultaneous detection and quantification of abundant and trace sugar nucleotides), (ii) insensitive to non-sugar nucleotide contaminants (which often confound UV detection methods), and (iii) capable of the unambiguous identification of 10 sugar nucleotides (UDP-Glc, UDP-Galp, UDP-Galf, UDP-GlcNAc, UDP-Rha, UDP-Xyl, GDP-Glc, GDP-Man, GDP-Fuc, and UDP-GlcA). To satisfy the first two criteria, we elected to use an LC-ES-MS/MS system in MRM mode. MRM methods are highly selective for the detection of compounds of interest in complex mixtures, where they may be relatively minor components. In essence, a compound is detected based upon both the mass of the whole molecule and the mass of one or more of its fragments. Thus, only when a molecular ion gives rise to a specific fragment ion is the ion recorded and assigned. To establish the so-called MRM transitions for each sugar nucleotide, sugar nucleotide standards were analyzed by negative ion ES-MS to find the \([M-H]^-\) precursor ion, and that ion was then selected and subjected to collision-induced dissociation, yielding an ES-MS/MS product ion spectrum that was used to choose a prominent product ion.

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TABLE 3. Comparison of sugar nucleotide levels, concentrations, demands, and minimum turnover rates in the three trypanosomatids

| Sugar nucleotide | T. brucei bloodstream form | T. cruzi epimastigote form | L. major promastigote form |
|------------------|---------------------------|---------------------------|----------------------------|
| **Pool size**    |                           |                           |                            |
| **T. brucei**    | 8 x 10^7 cells            | 10 x 10^7 cells           | 6 x 10^7 cells             |
| **T. cruzi**     | 2 x 10^8 cells            | 3 x 10^8 cells            | 1 x 10^8 cells             |
| **L. major**     | 5 x 10^7 cells            | 1 x 10^8 cells            | 2 x 10^7 cells             |

| Sugar nucleotide | T. brucei | T. cruzi | L. major |
|------------------|-----------|----------|----------|
| **Pool size**    |           |          |          |
| **T. brucei**    | 107 cells | 107 cells | 107 cells |
| **T. cruzi**     | 107 cells | 107 cells | 107 cells |
| **L. major**     | 107 cells | 107 cells | 107 cells |

**Amount (pmoles/10^7 cells)**

| Sugar nucleotide | T. brucei | T. cruzi | L. major |
|------------------|-----------|----------|----------|
| **Pool size**    |           |          |          |
| **T. brucei**    |           |          |          |
| **T. cruzi**     |           |          |          |
| **L. major**     |           |          |          |

**Concentration (pmoles/10^7 cells)**

| Sugar nucleotide | T. brucei | T. cruzi | L. major |
|------------------|-----------|----------|----------|
| **Pool size**    |           |          |          |
| **T. brucei**    |           |          |          |
| **T. cruzi**     |           |          |          |
| **L. major**     |           |          |          |

**Demand (pmoles/10^7 cells)**

| Sugar nucleotide | T. brucei | T. cruzi | L. major |
|------------------|-----------|----------|----------|
| **Pool size**    |           |          |          |
| **T. brucei**    |           |          |          |
| **T. cruzi**     |           |          |          |
| **L. major**     |           |          |          |

**Turnover (min)**

| Sugar nucleotide | T. brucei | T. cruzi | L. major |
|------------------|-----------|----------|----------|
| **Pool size**    |           |          |          |
| **T. brucei**    |           |          |          |
| **T. cruzi**     |           |          |          |
| **L. major**     |           |          |          |

**DISCUSSION**

**A quantitative method for sugar nucleotide analysis.** There are several methods available in the literature to extract and analyze sugar nucleotides from cells (55, 92, 94, 107, 115). These methods use a variety of different techniques for the separation, detection, and characterization of sugar nucleotides. In some of the methods, sugar nucleotides are detected using UV absorbance at 260 nm and quantified based upon standard curves. In other methods, MS and nuclear magnetic resonance are used either online or offline to structurally identify and characterize sugar nucleotides or biosynthetic intermediates. However, none of the existing methods combines the power of MS for definitive metabolite identification with a quantitative analysis. For these reasons, we felt that there was scope for producing an LC-ES-MS/MS method that would be able to quantify multiple sugar nucleotides extracted from different organisms. A major benefit of the MRM-based approach that we took is that it can provide identification and quantification of coeluting sugar nucleotides as long as they have different masses. This is important because many of the existing methods that quantify sugar nucleotides are hampered by a lack of HPLC separation between these closely related metabolites. The MRM-based approach is also very sensitive because the mass spectrometer spends time looking for ions of interest rather than scanning an entire mass range. For these reasons, we believe that the MRM method is a good tool for detecting and quantifying low-abundance sugar nucleotides, such as UDP-Galg, UDP-Xyl, UDP-Ara, and GDP-Fuc, from relatively small culture volumes (10 to 100 ml). The method has already proved useful in characterizing sugar nucleotide levels for two UDP-Gal 4'-epimerase-deficient cell lines (62, 120) and will be applied to other trypanosome mutants to monitor the effects on sugar nucleotide levels.

**Overview of the results.** Qualitatively, the sugar nucleotide contents of the three trypanosomatids obtained with the LC-ES-MS/MS method agree well with the monosaccharide contents of known trypanosomatid glycoconjugates (Table 1) and/or the presence of known or candidate sugar nucleotide biosynthesis enzymes (Fig. 2 and Table 4). In some cases, the metabolite identification data enable us to improve the annotations of genes likely to be involved in sugar nucleotide biosynthesis (Table 4). The concentrations of the various sugar nucleotides measured in this study reflect their steady-state standard deviation for triplicate analyses is typically ±1%, the deviations from the means presumably represent errors introduced by the extraction part of the method.

Representative MRM traces for bloodstream form T. brucei (Fig. 1A), promastigote form L. major (Fig. 1B) and epimastigote form T. cruzi (Fig. 1C) cells show the sugar nucleotides that we were able to detect in these organisms. Five sugar nucleotides were detected in all the organisms analyzed (UDP-Glc, UDP-Galp, GDP-Man, UDP-GlcNAc, and GDP-Fuc) together with the UDP-Galp and a GDP-pentose (assumed to be GDP-Ara) in L. major and UDP-Rha, UDP-Xyl, and UDP-Galp in T. cruzi. The yield of each sugar nucleotide (pmoles per 10^7 cells) and the estimated cellular concentration, based on cell volume and assuming free distribution of the metabolites, are shown in Table 3.
FIG. 1. Representative LC-ES-MS/MS MRM chromatograms of sugar nucleotide extracts. Sugar nucleotides were obtained from bloodstream form *T. brucei* (A), promastigote form *L. major* (B), and epimastigote form *T. cruzi* (C). The UDP-Gal peaks are amplified 15-fold. The GDP-Glc peaks are the internal standard added to allow quantification. Two peaks appear in the GDP-glucose channel due to “bleed-through” of GDP-Man, which undergoes the same mass transition.
pool sizes and do not appear to reflect the demand for these sugars (Table 3). In the following sections, we review the data for each sugar nucleotide pool obtained in this study and integrate it with our current knowledge of glycobiology and genomes of T. brucei, T. cruzi, and L. major.

GDPM-Man biosynthesis and metabolism. The presence of GDP-Man in T. brucei, T. cruzi, and L. major is consistent with the presence of H9251-D-Man and H9252-D-Man in glycoprotein N-linked glycans in all three species, the presence of H9251-D-Man in free and/or protein-linked GPIs in all three species, and the presence of H9251-D-Man in LPG and the P-linked glycans of the PPGs of L. major and NETNES of T. cruzi. GDP-Man is the direct donor substrate for many mannosyltransferases (e.g., for the synthesis of the Man5GlcNAc2-PP-Dol intermediate of protein N glycosylation), whereas for others it is an obligate intermediate, via Dol-P-Man synthetase, to the Dol-P-Man donor (e.g., for GPI anchor biosynthesis and the conversion of Man5GlcNAc2-PP-Dol to Man9GlcNAc2-PP-Dol in T. brucei and T. cruzi). GDP-Man is also the precursor of GDP-Fuc (see below).

The de novo pathway of GDP-Man biosynthesis is from glucose-6-phosphate, i.e., Glc-6-P → Fru-6-P → Man-6-P → Man-1-P → GDP-Man, and candidate or characterized genes for all of these steps can be found in the three genomes. However, the ability to biosynthetically radiolabel T. brucei, T. cruzi, and L. major with [3H]Man (84, 111, 118) illustrates that a salvage pathway, presumably via the hexokinase (Man3Man-6-P), also exists.

Despite the huge demand for Man by all three parasites (Table 3), the steady-state levels of GDP-Man are low (8 to 55 μM) and the minimum rates of turnover of the GDP-Man pools during logarithmic growth, based on the estimated copy numbers of Man residues per parasite, are in the orders of seconds for bloodstream form T. brucei and promastigote form T. brucei and epimastigote form T. cruzi (Table 3). The low steady-state concentrations of the cellular GDP-Man pools and the very high rates of flux through them suggests that inhibition of GDP-Man biosynthesis would have rapid and profound effects on glycoconjugate biosynthesis, and consequently on viability and/or virulence, in these organisms. Certainly, GDP-Man and Dol-Man biosynthesis may be assumed to be essential in bloodstream form T. brucei, where disrupting GPI anchor biosynthesis (which is dependent on Dol-Man made from GDP-Man) is known to be lethal for the parasite (15, 26, 60, 74). Since Man is present in every known type of trypanosomatid glycoconjugate carbohydrate chain, with the exception of the O-linked side chains of the T. cruzi mucins, GDP-Man biosynthesis has potential as a therapeutic target against all three parasites, so long as parasite-selective inhibitors can be found.

Mannose metabolism has been studied in any detail only for the leishmania. Surprisingly, the promastigote form of the organism can survive in culture in the complete absence of mannose metabolism. Thus, promastigote mutants null for phosphomannose isomerase (Fru-6-P → Man-6-P), phosphomannose mutase (PMM; Man-6-P → Man-1-P), GDP-Man pyrophosphorylase (GDPMP; Man-1-P → GDP-Man), Dol-Man synthetase (GDP-Man → Dol-Man), and a Golgi GDP-Man transporter (LPG2) were all viable in culture despite the devastating effects these mutations had on the mutants’ cell surface molecular architecture (32–34, 48, 110). Whereas the phosphomannose isomerase and Dol-Man synthetase mutants retained the ability to infect macrophages and mice, albeit with reduced virulence, the PMM and GDPMP mutants could not infect macrophages in vitro or in vivo. These results showed that GDP-Man is essential for reasons other than just supplying Dol-Man, upon which N glycosylation and GPI anchor, GIPL, and LPG biosynthesis are dependent. The recently characterized β-mannan storage polysaccharide and/or its biosynthetic intermediates appear to be the
almost indistinguishable from that of the human enzyme, and this crystal structure of drug targets against the leishmania. However, the high-resolution parasite virulence in the host suggests that these may be good thesis (72). Under such conditions, a mannose storage polysaccharides (93, 104). The extremely rapid turnover of GDP-Man pools is thus likely to make the search for parasite-selective drug leads difficult (53). Interestingly, despite its structural and enzymatic similarities to L. major PMM, human PMM cannot complement an L. major PMM null mutant for virulence. However, the GDPMP has been activity based on the Ara-1-P step (100, 101)

| Step no. | Enzyme name | Enzyme no. | Putative/characterized trypanosomatid homologues |
|----------|-------------|------------|--------------------------------------------------|
| 1        | Hexokinase  | EC 2.7.1.1 | Tb10.70.5820 Tb10.70.5800 LmjF12.0240 LmjF12.0250 |
| 2        | Glucose-6-phosphate isomerase | EC 5.3.1.9 | Tb927.1.3830 LmjF12.0530 Tb10.40705350629520 |
| 3        | Glucosamine-fructose-6-phosphate aminotransferase | EC 2.6.1.16 | Tb297.5560 LmjF06.0950 Tb10.40705351030320 |
| 4        | Glucosamine-phosphate N-acetyltransferase | EC 2.3.1.4 | Tb11.01.2886 LmjF28.3005 Tb10.40705350883120 |
| 5        | Phosphoacetylglucosamine mutase | EC 5.4.2.3 | Tc00.10470535099650 Tc00.10470535078375 |
| 6        | UDP-N-acetylglucosamine pyrophosphorylase | EC 2.7.7.23 | Tb11.01.6410 LmjF32.1380 Tb10.40705350370710 |
| 7        | Phosphomannose isomerase | EC 5.3.1.8 | Tc00.10470535117171 |
| 8        | Phosphomannomutase | EC 5.4.2.8 | Tb10.70.2050 LmjF23.0110 Tb10.40705351094710 |
| 9        | Mannose-1-phosphate guanylyltransferase | EC 2.7.7.13 | Tb10.70.2050 LmjF23.0110 |
| 10       | GDP-mannose 4,6-dehydratase | EC 4.2.1.46 | Tb10.40705350863790 Tb10.40705351091130 |
| 11       | GDP-1-fucose synthetase | EC 1.1.1.271 | Tb10.39890900 Tb10.40705350352150 |
| 12       | Phosphoglumutase | EC 5.4.2.2 | Tb10.40705350863790 Tb10.40705351091130 |
| 13       | UTP glucose-1-phosphate uridylyltransferase | EC 2.7.1.1 | Tb10.40705350352150 |
| 14       | UDP-galactose 4-epimerase | EC 5.1.3.2 | Tb10.40705350863790 Tb10.40705351091130 |
| 15       | UDP-galactopyranose mutase | EC 5.4.9.9 | Tb10.40705350863790 Tb10.40705351091130 |
| 16       | Galactokinase | EC 2.7.1.6 | Tb10.40705350863790 Tb10.40705351091130 |
| 17       | UTP hexose-1-phosphate uridylyltransferase or UDP-glucose hexose-1-phosphate uridylyltransferase | EC 2.7.7.10 or EC 2.7.7.12 | |
| 18       | UDP-glucose 4,6-dehydratase | EC 4.2.1.46 | |
| 19       | UDP-4-keto-6-deoxyglucose reductase | EC 1.1.1.133 | Tb10.40705350863790 Tb10.40705351091130 |
| 20       | UDP-glucose 6-dehydrogenase | EC 1.1.1.22 | Tb10.40705350863790 Tb10.40705351091130 |
| 21       | UDP-glucuronate decarboxylase | EC 4.1.1.35 | Tb10.40705350863790 Tb10.40705351091130 |
| 22       | Pathway unknown | | |
| 23       | Fucose (arabinose) kinase | EC 2.7.1.52 or EC 2.7.1.54 | |
| 24       | Arabinose-1-phosphate guanylyltransferase | Not assigned | |

a Step numbers refer to those shown in Fig. 2.
b Gene numbers in boldface have been functionally characterized, those underlined are as assigned in GeneDB, and those in boldface and underlined are assigned in this paper through BLAST searches with functionally characterized orthologues from other organisms.

gene encoding PMM has a C-terminal PTS1 signal sequence (SKL) that may direct it into the glycosome (81), and mislocation of the human PMM might be the reason. The GDPMP has been expressed and biochemically characterized (18), but no crystal structure is yet available.

**UDP-GlcNAc biosynthesis and metabolism.** The presence of UDP-GlcNAc in *T. brucei*, *T. cruzi*, and *L. major* is consistent with the presence of GlcNAc in glycoprotein N-linked glycosylation.
in all species and in O-linked glycans in *T. cruzi*. In addition, GlcN, derived from GlcNAc by de-N-acetylation (24), is present in all species in protein-linked and free GPI structures, including *L. major* LPG. In *T. brucei*, GlcNAc is found in LacNac repeats of Galpβ1-4GlcNac. These poly-LacNac structures are found as part of giant N-linked glycans throughout the flagellar pocket and endosomal/lysosomal system of the bloodstream form (5, 76) and as side chains of the procyclin GPI anchor and GIPLs in the procyclic form (73, 96, 116) of the organism.

Bioinformatic analysis suggests that UDP-GlcNAc, the direct donor for all GlcNAc transferases, is made by the conventional eukaryotic de novo route from glucose-6-phosphate, i.e., Glc-6-P → Fru-6-P → GlcN-6-P → GlcNAc-6-P → GlcNAc-1-P → UDP-GlcNAc. However, the ability to biosynthetically radiolabel *T. brucei* and *L. major* glycoconjugates with \(^{3}H\)GlcN (30, 91) shows that a salvage pathway, presumably via the action of hexokinase (GlcN → GlcN-6-P), also exists. Most likely, the former pathway is the most important in vivo, since free GlcN is not an abundant sugar in either mammals or insects and, at least in *T. brucei*, its N-acetyl derivative (GlcNAc) is not taken up (6). Since GlcNAc and/or GlcN is present in every known type of trypanosomatid glycoconjugate carbohydrate chain, with the exception of the side chains of leishmania and *T. cruzi* PPGs, UDP-GlcNAc biosynthesis has potential as a therapeutic target against all three parasites, so long as parasite-selective inhibitors can be found. The essential nature of UDP-GlcNAc pyrophosphorylation to bloodstream form *T. brucei* has recently been demonstrated by construction of a conditional null mutant (M. Stokes and M. A. J. Ferguson, unpublished data).

UDP-GlcNAc is relatively abundant in all three parasites (ranging from 150 μM in bloodstream form *T. brucei* to 410 μM in *T. cruzi* epimastigotes), and the minimum rates of turnover of the UDP-GlcNAc pools during logarithmic growth, based on the estimated copy numbers of GlcNAc residues per parasite, are in the orders of tens of minutes to hours (Table 3). The steady-state levels of UDP-GlcNAc between the parasites do not appear to reflect the demand for GlcNAc between the organisms.

**UDP-Glc biosynthesis and metabolism.** UDP-Glc is also found in all three parasites and is the glucosyl donor for the unfolded glycoprotein glucosyltransferase (UGGT) involved in glycoprotein quality control in the endoplasmic reticulum (42, 117). UGGT is known to function in all three parasites (17, 51, 68, 80). UDP-Glc is also the presumed donor for the synthesis of base J (β-D-glucosylhydroxymethyluracil), a rare deoxynucleotide found in telomere-proximal DNA in the bloodstream form of *T. brucei* and in the leishmania and *T. cruzi* (121). UDP-Glc is also presumably the precursor of UDP-Rha and UDP-Xyl in *T. cruzi* and is the obligate precursor of UDP-Galp in *T. brucei* and *T. cruzi* (see below). The UDP-Glc pyrophosphorylase from *L. major* has been cloned, expressed, and enzymatically and structurally characterized (57, 109).

The demand for UDP-Glc must large, because it includes the flux through this pool to UDP-Galp via UDP-Glc 4′-epimerase (see below). However, since we know neither the average numbers of UGGT/glucosidase II-mediated cycles of transient glucosylation/deglucosylation involved in endoplasmic reticulum glycoprotein quality control in these organisms nor the rates of turnover of base J, is difficult to assess the total demand. Nevertheless, the steady-state UDP-Glc pool sizes are relatively large (110 to 540 μM) for all of the cells analyzed, probably because the substrates for the UDP-Glc pyrophosphorylase (i.e., Glc-1-P and UTP) are relatively abundant metabolites.

**UDP-Galp biosynthesis and metabolism.** The presence of UDP-Galp in *T. brucei*, *T. cruzi*, and *L. major* is consistent with the presence of Galp in many of their glycoconjugates (Table 1). Furthermore, UDP-Galp is also the obligate precursor for UDP-Galf in *T. cruzi* and *L. major* (see below). The only route to UDP-Galp in bloodstream and procyclic-form *T. brucei* and epimastigote form *T. cruzi* is via the epimerization of UDP-Glc, because their hexose transporters are unable to transport Gal (9, 112). The UDP-Glc 4′-epimerase (GalE) responsible for this interconversion is essential in both life cycle stages of *T. brucei* (96, 97, 120) and appears to be essential for *T. cruzi* epimastigotes (62), suggesting that this enzyme may be a good therapeutic target against African trypanosomiasis and possibly Chagas’ disease.

With respect to therapeutic potential, the need for selective inhibition of trypanosome UDP-Glc 4′-epimerase over the human enzyme may be less acute for than for, say, enzymes of GDP-Man, UDP-GlcNAc, and UDP-Glc biosynthesis. This is because (i) human cells have salvage pathways for UDP-Galp biosynthesis (indeed, Gal-1-P and UDP-Galp accumulate in GalE null mammalian cells [103]) and (ii) although defects in UDP-Glc biosynthesis (indeed, Gal-1-P and UDP-Galp accumulate in GalE null mammalian cells [103]) and (ii) although defects in

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putative \( L. \) \( major \) galactokinase gene is functional, although we were unable to find an obvious galactose-1-phosphate uridylyltransferase (EC 2.7.7.10) or UDP-Glc hexose-1-phosphate uridylyltransferase (EC 2.7.7.12) that might complete a UDP-Gal salvage pathway in this organism. Interestingly, \( T. \) \( brucei \) has a galactokinase pseudogene that might have degenerated when the organism lost its ability to take up Gal. Putative galactokinase genes also exist in \( T. \) \( cruzi \), even though this organism also cannot take up Gal. It is possible that one or more of these may be involved in salvage pathways for other sugars in this organism.

**UDP-Gal biosynthesis and metabolism.** Both \( L. \) \( major \) and \( T. \) \( cruzi \) express abundant Gal-containing molecules, and UDP-Gal is found in both organisms. UDP-Gal is made from UDP-Galp by the action of UDP-Gal mutase, and \( L. \) \( major \) and \( T. \) \( cruzi \) contain functional and putative UDP-Gal mutase genes, respectively (7, 12). \( T. \) \( brucei \) lacks a putative UDP-Gal mutase gene and contains neither UDP-Gal nor Gal-containing glycoconjugates.

The steady-state pool sizes of UDP-Gal in \( L. \) \( major \) and \( T. \) \( cruzi \) are extremely small, but they are in line with the reported equilibrium constant of the \( Escherichia \) \( coli \) UDP-Gal mutase (75). Thus, the ratio of UDP-Galp to UDP-Gal in both organisms is close to 14:1. Given the high levels of Gal in the glycoconjugates of both organisms, the turnover rate of the UDP-Gal pool must be in the order of minutes. Interestingly, when one allele of \( GalE \) is replaced in epimastigote form \( T. \) \( cruzi \), the cells no longer express significant amounts of the Galp-rich GPI anchored mucins but retain most of their Gal-containing GPIs (62). In order to achieve this preferential retention of Gal metabolism, the pool size of UDP-Galp (and therefore that of UDP-Galp) is not reduced significantly. Presumably, the flux through the UDP-Galp pool to UDP-Galp transporters/Galp transferases is reduced to maintain flux through the UDP-Galp pool.

The lack of Gal in mammals has led to the suggestion that the biosynthesis and metabolism of UDP-Galp might be potential therapeutic targets against the leishmanias and \( T. \) \( cruzi \), as well as other microbial pathogens (22, 85). However, a recent report by Kleecka and colleagues shows that \( L. \) \( major \) UDP-Galp mutase null mutants are still infectious to mice, albeit with reduced virulence (54). In the case of \( T. \) \( cruzi \), anti-Gal antibodies have been reported to inhibit the invasion of host cells (19), but the relative importance of Gal, which is most abundant in the insect-dwelling epimastigote stage, in parasite survival and/or propagation in the mammalian host is unknown.

**UDP-Xyl and UDP-Rha biosynthesis and metabolism.** Of the three trypanosomatids, only \( T. \) \( cruzi \) is known to express glycoproteins that contain Xyl and Rha and, consistent with this distribution, UDP-Xyl and UDP-Rha appear only in \( T. \) \( cruzi \). The sugars are found as \( \beta-D-Xyl \) and \( \alpha-L-Rha \) in the novel low-abundance Ser/Thr-phosphate-linked carbohydrate chains recognized by the \( T. \) \( cruzi \)-specific monoclonal antibody WIC29.26 (3, 29, 40), which are found principally, but not exclusively, on gp72, a glycoprotein associated with flagellar attachment to the cell body (20). UDP-Xyl is the only known xylose sugar nucleotide in nature, and its biosynthesis goes from UDP-Glc via UDP-glucuronic acid (UDP-GlcUA). Genes for both of these steps can be found in the \( T. \) \( cruzi \) genome, and the intermediate sugar nucleotide (UDP-GlcUA) was also found in our analyses (Table 3). Whether UDP-GlcUA has any function other than as an intermediate on the pathway to UDP-Xyl is unclear at the moment, but the \( T. \) \( cruzi \) genome does not contain any identifiable putative glucuronosyltransferases.

\( D-Rhamnose \) is found both as UDP-Rha, described for plants and made from UDP-Glc (52, 79, 123), and as dTDP-Rha, described for bacteria and made from dTDP-Glc (35). The results reported here show that \( T. \) \( cruzi \) makes UDP-Rha, presumably from UDP-Glc, rather than dTDP-Rha from dTDP-Glc. We therefore suggest corrections to the GeneDB annotations, which suggest a dTDP-Rha pathway (Table 4). This is a common problem in the genome databases, where eukaryotic genes encoding dehydratases and epimerase/reductases likely to be involved in GDP-Fuc or UDP-Rha biosynthesis have, through automated annotation, been assigned to prokaryote-specific dTDP-Rha biosynthesis. In the plant \( Arabidopsis \) \( thaliana \), the three enzymatic activities of UDP-Rha biosynthesis are located in a single polypeptide (RHM2/MUM4), with the UDP-Glc 4,6-dehydratase activity located in the N-terminal domain (residues 1 to 370) and the UDP-4-keto-6-deoxy-Glc epimerase and UDP-4-keto-Rha reductase activities located in the C-terminal domain (residues 371 to 667) (79, 123). In \( T. \) \( cruzi \), these domains appear to belong to separate polypeptides. Presumably, one of the several putative \( T. \) \( cruzi \) NDP-sugar 4,6-dehydratases performs the first step, and the products of one or both of two closely related genes that show significant homology to the C-terminal domain of RHM2/MUM4 perform the epimerase and reduction steps (Table 4).

The presence and maintenance of multistep de novo biosynthetic pathways to UDP-Xyl and UDP-Rha in \( T. \) \( cruzi \) suggest that these sugars may play an important role in the biology of this organism. It is known that gp72 is important for flagellar adhesion in epimastigote form \( T. \) \( cruzi \) (20) and that gp72 null parasites have reduced virulence in both the insect vector and the mammalian host (10). However, our knowledge of gp72 carbohydrate structure does not extend beyond the epimastigote form, and it is clear that gp72 glycosylation and the expression of the WIC29.26 epitope is stage specific (40, 41). Thus, at the moment it is difficult to deduce the precise function(s) of Xyl and Rha metabolism in \( T. \) \( cruzi \).

**GDP-Ara biosynthesis and metabolism.** Only \( L. \) \( major \) and \( C. \) \( fasciculata \) are known to express arabinose in the form of \( D-arabinose \). This sugar is found in \( L. \) \( major \) LPG and in the related \( C. \) \( fasciculata \) lipoarabinogalactan (66, 102). Correspondingly, GDP-Ara was found only in \( L. \) \( major \) in this study.

The source of GDP-\( D-arabinose \) in \( L. \) \( major \) is not fully understood. It is known that \( L. \) \( major \) and \( C. \) \( fasciculata \) can convert \( D-Glc \) to \( D-Ara \) via the loss of the Glc C-1 carbon atom and that they can also take up exogenous Ara (100, 101). A 125-kDa \( C. \) \( fasciculata \) \( D-arabinose-1-kinase \) activity has been characterized but not cloned (100), suggesting that the route is Glc → Ara → Ara1-P → GDP-Ara (Fig. 2). The \( C. \) \( fasciculata \) \( D-arabinose-1-kinase \) also has fucose-1-kinase activity (\( D-Ara \) and \( L-Fucp \) differ by only a methyl group), and we suggest that the two genes annotated as putative \( L. \) \( major \) fucose kinase genes in GeneDB should be reclassified as putative arabinose/fucose kinase genes (Table 4). The proposed route to GDP-Ara re-
quires a GDP-Ara pyrophosphorylase activity, but no candidate genes (e.g., homologues of mammalian GDP-Fuc pyrophosphorylase genes) are apparent in the L. major genome.

In L. major promastigotes, the proportion of phosphosaccharide repeats with β-α-D-Arap-terminating side chains is under developmental control, such that levels of Ara are low in log-arithmically growing procyclic promastigotes and significantly higher in metacyclic promastigotes (67, 87). Studies on the arabinosyltransferases in L. major have led to the suggestion that the control of arabinosylation may be at the level of GDP-Ara availability rather than at that of arabinosyltransferase expression (23). Certainly, GDP-Ara levels were low in the procyclic promastigotes studied here.

**GDP-Fuc biosynthesis and metabolism.** The fucose donor GDP-Fuc was found in all three trypanosomatids, even though Fuc has been identified thus far only in the aforementioned novel low-abundance Ser/Thr-phosphate-linked carbohydrate chains of T. cruzi gp72 (3, 29, 40). On the other hand, the presence of GDP-Fuc in T. brucei is not so surprising, given that the genomes of T. cruzi and T. brucei both contain homologues of the two enzymes, GDP-Man 4,6-dehydratase and the combined GDP-4-keto-6-deoxy-Man 3,6-epimerase and GDP-4-keto-Fuc reductase, needed for de novo synthesis of GDP-Fuc from GDP-Man (Fig. 2). T. brucei also contains homologues (Fla-1 and Fla-2) of the fucosylated T. cruzi Fla-1 and Fla-2 are also involved in flagellar Fuc from GDP-Man (Fig. 2).

4-keto-Fuc reductase, needed for de novo synthesis of GDP-Fuc from GDP-Man, and Fuc has been identified thus far only in the aforementioned genomes (23). Certainly, GDP-Ara levels were low in the procyclic promastigotes of T. cruzi and Leishmania major. Ph.D. thesis, University of Dundee, Dundee, Scotland.

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