THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 3, Issue of February 10, pp. 909-916, 1980
Printed in U.S.A.

Purine Phosphoribosyltransferases from *Leishmania donovani**

(Received for publication, August 17, 1979)

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Three distinct enzymes which catalyze phosphoribosyl transfer to purines were found in promastigotes of the pathogenic protozoan, *L. donovani*. One enzyme showed activity with hypoxanthine and guanine (Hyp-Gua phosphoribosyltransferase), a second with adenine (Ade phosphoribosyltransferase), and a third with xanthine (Xan phosphoribosyltransferase). Relative heat stabilities at 37°C were Hyp-Gua phosphoribosyltransferase > Xan phosphoribosyltransferase > Ade phosphoribosyltransferase. The enzymes were separated by Sephadex G-100 chromatography in Tris/Mg buffer. Particle weights were 110,000 for the Hyp-Gua enzyme, 54,000 for the Xan enzyme, and 25,000 for the Ade enzyme. When chromatographed under the same conditions except for the presence of 1 mM PP-ribose-P, the three enzymes were not well separated, displaying particle weights of 60,000, 62,000, and 54,000, respectively. Isoelectric focusing yielded pI values of 7.5 for the Xan enzyme and 7.0 for the Hyp-Gua enzyme. Ade phosphoribosyltransferase was inactivated by this procedure. Ade phosphoribosyltransferase and Hyp-Gua phosphoribosyltransferase were purified 46- and 110-fold, respectively, by affinity chromatography on agarose-AMP and agarose-GTP columns. The structure-activity relationships of both enzymes were investigated by determining the kinetic constants for a variety of purines and purine analogues. For comparison, kinetic constants were also determined for Hyp-Gua phosphoribosyltransferase purified from human erythrocytes. Pyrazolo(3,4-*d*)pyrimidines were more efficient substrates for the parasite Hyp-Gua phosphoribosyltransferase than for the human enzyme, while the converse was found with 8-azapurine analogues.

Purine phosphoribosyltransferases catalyze the condensation of the 5-phosphoribosyl moiety derived from PP-ribose-P[1] with purine bases to form purine 5′-ribonucleotides. These enzymes are the major metabolic pathway for the salvage of preformed purines and are widely distributed in nature. Fundamental differences between some bacterial and mammalian purine phosphoribosyltransferases have been observed (1, 2).

Most parasitic protozoa lack the ability to synthesize purines *de novo* and are dependent upon their host for purines (3). Although cell-free extracts of several protozoa have been found to catalyze phosphoribosyl transfer to adenine (4–6), hypoxanthine, and guanine (4–7), there is little information about the multiplicity or specificity of purine phosphoribosyltransferases from protozoa. In particular, the enzymatic basis for phosphoribosyl transfer to xanthine has not been investigated.

Leishmania donovani is the causative agent of visceral leishmaniasis or kala azar in man. Purine phosphoribosyltransferases have not been previously described from this parasite. Like most other pathogenic protozoa, it is an obligate scavenger of preformed purines (8). When promastigotes of *L. donovani* are exposed to the hypoxanthine analogue, allopurinol, growth is inhibited (9) and large quantities of allopurinol ribonucleoside 5′-monophosphate accumulate (10). In contrast, mammalian cells accumulate only minute quantities of this ribonucleotide (11). The possibility exists that this difference is the result of differences in the substrate specificities of the purine phosphoribosyltransferases.

This report describes the separation of three distinct purine phosphoribosyltransferases from promastigotes of *L. donovani*: one specific for Ade, another for Hyp-Gua, and a third for Xan. The latter enzyme was not studied in detail due to its instability. The former were further purified and their substrate specificities were examined. A direct comparison of the substrate specificities of the Hyp-Gua phosphoribosyltransferase from *L. donovani* and from human erythrocytes was made.

EXPERIMENTAL PROCEDURES

Materials—Na_4PP-ribose-P, bovine serum albumin (Fraction V), orotic acid, purine, purine ribonucleoside, 6-methylpurine, 6-methylpurine ribonucleoside, isoguanine sulfate, 8-azaadenine, pterin, 5-aminoimidazole-4-carboxamide·HCl, 1-ribosyl-5-aminoimidazole-4-carboxamide, and xanthosine 5′-phosphate were purchased from Sigma. Agarose-hexane-AMP (type 2), agarose-hexane-GTP (type 4), orotate phosphoribosyltransferase/OMP decarboxylase, adenosine 5′-phosphate, guanine, guanosine 5′-phosphate, hypoxanthine, inosine 5′-phosphate, 6-mercaptopurine ribonucleoside 5′-phosphate, and xanthine were purchased from P-L Biochemicals. Phenylmethylsulfonyl fluoride was purchased from Calbiochem. 4-Aminopteridine was purchased from Chemical Dynamics Corp. Ampholytes were purchased from LKB Instruments. Bovine milk xanthine oxidase (EC 1.2.3.2) and calf spleen purine nucleoside phosphorylase (EC 2.4.2.1) were purchased from Boehringer Mannheim Corp. Adenine and enzyme grade ammonium sulfate were purchased from Schwarz/Mann. All other purines and purine ribonucleosides and their analogues were synthesized in these laboratories according to published procedures.

Enzyme Assays—All enzyme activities were assayed spectrophotometrically at 25°C. The rate of change in absorbance resulting from the conversion of substrates to products was monitored at the appropriate wavelength using a Gilford 250 recording spectrophotometer at a full scale setting in the range of 0.02 to 0.1 absorbance units. Final assay mixtures had a total volume of 0.4 ml in a semimicro cuvette having a 10-mm pathlength. A 2 × 4 mm light aperature was used. Reactions were started by the addition of enzyme. The observed rate in the presence of substrate (purine base, nucleoside, or nucleotide) was corrected by subtracting the rate observed in the absence of substrate. In most cases, the latter was negligible. One unit of enzyme

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[1] The abbreviations used are: Ade phosphoribosyltransferase = adenine phosphoribosyltransferase (EC 2.4.2.7); Caps = 3-(cyclohexylamino)propanesulfonic acid; Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hyp-Gua phosphoribosyltransferase = hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); Pipes = 1,4-piperazine-*N,N′*-bis(2-ethanesulfonic acid); PP-ribose-P = 5-phosphoribosyl-1-pyrophosphate; Xan phosphoribosyltransferase = xanthine phosphoribosyltransferase.

activity is defined as that amount of enzyme which will catalyze the conversion of 1 μmol of substrate to product per min at 25°C. All specific activities were calculated on the basis of the protein content before the addition of the stabilizer, bovine serum albumin.

Phosphoribosyltransferase Assays—The final concentration of components in assay mixtures, unless otherwise specified, was 100 mM Tris-HCl, 5 mM $MgSO_4$, Na_4PP-ribose-P (2 mM when measuring enzyme levels, 1 mM when determining kinetic constants), 0.1 mM adenine, hypoxanthine, or xanthine or 0.04 mM guanine. The final pH of assay mixtures was 7.4 when hypoxanthine, xanthine, or guanine was used as substrate. When adenine was used as substrate, enzyme levels were measured at pH 9.2 and kinetic constants were determined at pH 7.4. The Δε values for the conversion of purines and purine analogues to their respective 5′-ribonucleotides are listed in Table I.

Assays for Interfering Enzyme Activities—PP-ribose-P was removed from each enzyme sample before it was assayed for these enzyme activities by passing 1 ml of the enzyme sample down a Sephadex G-25 column (0.8 × 13 cm) which had been equilibrated at 4°C with 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM $MgSO_4$.

All assay mixtures contained 100 mM Tris-HCl and 5 mM $MgSO_4$ and had a final pH of 7.4. Adenase (EC 3.5.4.2) reaction mixtures also contained 0.1 mM adenine ($\Delta\epsilon = -5{,}700\ M^{-1}cm^{-1}$ at 265 nm). Guanase (EC 3.5.4.3) assay mixtures also contained 0.04 mM guanine ($\Delta\epsilon = -4{,}230\ M^{-1}cm^{-1}$ at 246 nm). AMP deaminase (EC 3.5.4.6) assay mixtures also contained 0.1 mM AMP ($\Delta\epsilon = -8{,}450\ M^{-1}cm^{-1}$ at 263 nm). Xanthine oxidase/dehydrogenase (EC 1.2.3.2, EC 1.2.1.37) assay mixtures also contained 1 mM potassium ferricyanide, 0.1 mM Na_2EDTA, and 0.1 mM xanthine ($\Delta\epsilon = -2{,}080\ M^{-1}cm^{-1}$ at 420 nm). Nucleotidase was assayed, with IMP as substrate, by enzymatically coupling the formation of the product, inosine, to the formation of uric acid using purine nucleoside phosphorylase and xanthine oxidase. The rate of increase in absorbance at 292 nm ($\Delta\epsilon = 11{,}500\ M^{-1}cm^{-1}$) resulting from the formation of uric acid was measured. These nucleotidase assay mixtures also contained 0.1 mM Na_4EDTA, 5 mM potassium phosphate (pH 7.4), 32 mM ammonium sulfate, 0.1 mM IMP, 0.5 units of purine nucleoside phosphorylase, and 0.02 unit of xanthine oxidase. These levels of coupling enzymes were determined to be optimal and not rate-limiting.

Other Determinations—Particle weight values were estimated from the elution volumes from a Sephadex G-100 column which was calibrated with proteins of known molecular weights (12). PP-ribose-P was quantitated using a coupled enzyme system consisting of orotate phosphoribosyltransferase and OMP decarboxylase as described by Kornberg *et al.* (13). Protein was quantitated using the Coomassie blue method with bovine serum albumin as the standard (14).

Enzyme Source—*L. donovani* promastigotes were generously provided by the laboratory of Dr. J. Joseph Marr of St. Louis University School of Medicine. The organisms were cultivated as previously described (15). Cultures were centrifuged at 3000 × *g* for 10 min. The cell pellet was suspended in 50 mM Hepes buffer, pH 7.4, which contained 5 mM $MgSO_4$, 1 mM PP-ribose-P, and 1 mM dithiothreitol. One milliliter of this buffer per g of wet cells was used. Aliquots of these cell suspensions were stored at −20°C. Unless otherwise mentioned all subsequent procedures were performed at 4°C.

Preparation of Cell Extracts—Frozen *L. donovani* promastigote cell suspensions (2 to 20 ml) were thawed at 25°C and diluted with one volume of 40 mM potassium phosphate buffer, pH 7.4, containing 10 mM $MgSO_4$ and 2 mM PP-ribose-P. The diluted suspension was homogenized for two 15-s periods, separated by a 30-s cooling period, with a Brinkmann Polytron at setting 10. After centrifugation at 100,000 × *g* for 30 min, the supernatant was desalted by passage down a Sephadex G-25 column (2.5 × 30 cm) previously equilibrated with 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM $MgSO_4$ and 1 mM PP-ribose-P. The desalted supernatant is referred to below as "cell extract."

Ammonium Sulfate Fractionation—The cell extract was brought to 40% saturation with saturated ammonium sulfate solution (4°C) and stirred for 30 min. After centrifugation at 45,000 × *g* for 30 min, the supernatant was brought to 80% saturation with solid ammonium sulfate (0.29 g/ml) and stirred overnight. This suspension could be stored for at least 3 months without any appreciable loss in purine phosphoribosyltransferase activities. After centrifugation as above, the pellet was dissolved in a minimal volume of the indicated buffer and this "40 to 80% ammonium sulfate fraction" was used in the experiments described below.

Purification of Hyp-Gua Phosphoribosyltransferase from Human Erythrocytes—Hyp-Gua phosphoribosyltransferase was partially purified from human erythrocytes using the first four steps of the procedure described by Holden and Kelley (16). The resulting preparation was applied to an agarose-GTP column (1 × 14 cm) previously equilibrated at 4°C with 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM $MgSO_4$, and 0.1 M KCl (Buffer A). The column was washed with Buffer A at a flow rate of 0.5 ml/min to remove any unbound proteins. Hyp-Gua phosphoribosyltransferase was eluted with Buffer A containing 5 mM Na_4PP-ribose-P. The specific activity of the eluted enzyme was 16.5 units/mg of protein with hypoxanthine as the substrate. Bovine serum albumin was added to the enzyme solution to a final concentration of 0.1 mg/ml. This solution was then brought to 80% saturation with ammonium sulfate. After stirring overnight, the suspension was centrifuged at 45,000 × *g* for 30 min. The pellet was dissolved in a minimal volume of 20 mM potassium phosphate, pH 7.4, containing 5 mM $MgSO_4$, 1 mM Na_4PP-ribose-P, and 1 mg/ml of bovine serum albumin. Aliquots of this concentrated enzyme solution were quick-frozen in a dry ice/acetone bath and stored at −70°C. This preparation contained no detectable Ade phosphoribosyltransferase, <0.1% (expressed as a percentage of the rate of phosphoribosyl transfer to guanine); xanthine oxidase/dehydrogenase, <0.3%; nucleotidase, <0.01%; or AMP deaminase, <0.01%. It did, however, contain guanase, 1.9%.

TABLE I

Absorbance changes resulting from the (phospho)ribosylation of purines and purine analogues at the position 9 of purine or its equivalent

Extinction coefficients of the purines and purine analogues listed below and their respective ribonucleosides or ribonucleoside 5′-phosphates were determined in 100 mM Tris-HCl, 5 mM $MgSO_4$, pH 7.4. The Δε was determined by subtracting the extinction coefficient of the purine or purine analogue from that of its 5′-phosphoribosyl or ribosyl derivative at the indicated wavelength.

Substrate	λ[a]	Δε
	nm	$mM^{-1}\ cm^{-1}$
Purine	275	1.6
6-Methylpurine	244	1.7
Adenine	256 (255)[b]	2.1 (3.3)[b]
2,6-Diaminopurine	258	3.6
6-Amino-2-hydroxypurine	300	3.2
Hypoxanthine	243	2.2
Guanine	255	4.2
Xanthine	250	3.9
6-Mercaptopurine	243	−1.7
4-Aminopyrazolo(3,4-*d*)pyrimidine	285	3.6
Allopurinol	273	1.6
6-Amino-4-hydroxypyrazolo(3,4-*d*)pyrimidine	255	7.5
4-Thiopyrazolo(3,4-*d*)pyrimidine	245	2.5
8-Azaadenine	297	3.4
8-Azahypoxanthine	281	4.7
8-Azaguanine	258	4.8
5-Aminoimidazole-4-carboxamide	244	1.7

[a] UV spectra were determined with a Beckman Acta III spectrophotometer. The wavelength was calibrated to an accuracy of ±0.5 nm using the deuterium emission lines at 486.1 and 656.2 nm.

[b] Parenthetical values were determined at pH 9.2.

RESULTS

Enzyme Levels—The purine phosphoribosyltransferase activities in a cell extract of *L. donovani* are shown in Table II. The activity was highest with guanine followed by the activities with hypoxanthine, adenine, and xanthine. When the pelleted cell debris obtained during the preparation of the cell extract was re-extracted, it was found to contain the following phosphoribosyltransferase activities expressed as a percentage of the total activity extracted from the promastigotes: adenine, 5%; guanine, 18%; hypoxanthine, 19%; and xanthine, 47%. These results indicated that the activity with xanthine was the most difficult to extract.

The cell extract was also assayed for those enzyme activities which might interfere with the phosphoribosyltransferase assays. As shown in Table II, only adenase was present at a level

TABLE II

Purine phosphoribosyltransferase and interfering enzyme activities in an extract of L. donovani promastigotes

The cell extract and enzyme assays are described under "Experimental Procedures."

Enzyme activity	Milliunits/mg protein
Phosphoribosyltransferase activity with:	
Guanine	140
Hypoxanthine	52
Xanthine	28
Adenine	45 (72)[a]
Adenase	25 (<0.2)[a]
Guanase	0.84
AMP deaminase	<0.08
Nucleotidase (IMP)	1.1
Xanthine oxidase/dehydrogenase	<0.3

[a] Parenthetical values were determined at pH 9.2 instead of pH 7.4. $\Delta\epsilon = -3.8\ M^{-1}\ cm^{-1}$ at pH 9.2 for adenine deamination.

which would significantly interfere with the assay for phosphoribosyl transfer to adenine. This difficulty was overcome by increasing the pH of the assay mixture from 7.4 to 9.2. As a result, the adenase activity was greatly reduced (Table II).

Enzyme Stability—The relative stabilities at 0°C of the phosphoribosyltransferase activities in the absence of magnesium and PP-ribose-P is shown in Fig. 1. The activities with hypoxanthine and guanine were the most stable; that with adenine was the least stable.

Magnesium and PP-ribose-P are known stabilizers of this class of enzymes. With the phosphoribosyltransferase activities from *L. donovani*, the most effective stabilization was achieved with a combination of both, although the activity with xanthine was only partially stabilized (Table III). Magnesium sulfate was an effective stabilizer of the activities with hypoxanthine and guanine but not with adenine or xanthine. Tetrasodium PP-ribose-P in the absence of magnesium partially stabilized the activity with adenine but not any of the other activities.

Gel Filtration—Fig. 2*A* shows the elution profile of the phosphoribosyltransferase activities in the presence of magnesium. Three peaks of activity were eluted, the first for hypoxanthine and guanine, the second for xanthine, and the last for adenine. Fig. 2*B* shows the markedly different profile in the presence of magnesium and PP-ribose-P. The activities with guanine and hypoxanthine were eluted in a single peak in both experiments with an average particle weight of 110,000 (±10%) in the absence of PP-ribose-P and 60,000 (±10%) in the presence of PP-ribose-P. The activity with xanthine was eluted at a volume corresponding to a particle weight of 54,000 (±10%) in the absence of PP-ribose-P and 62,000 (±10%) in the presence of PP-ribose-P. The activity with adenine was eluted at a volume corresponding to a particle weight of 25,000 (±10%) in the absence of PP-ribose-P and 54,000 (±10%) in the presence of PP-ribose-P.

When a sample of the concentrated human erythrocyte Hyp-Gua phosphoribosyltransferase containing bovine serum albumin was chromatographed on a Sephadex G-100 column under the conditions described in Fig. 2*A*, two peaks of Hyp-Gua phosphoribosyltransferase were eluted. One peak, which contained 24% of the activity applied, was eluted at the void volume (particle weight >130,000). The other peak, which contained 65% of the activity applied, was eluted at a volume corresponding to a particle weight of 74,000 (±10%). The addition of 1 mM PP-ribose-P to the elution buffer (conditions as in Fig. 2*B*) did not have any effect on this elution profile (data not shown).

Isoelectric Focusing—Fig. 3 shows the elution profile of the phosphoribosyltransferase activities from an isoelectric focusing column. The activities with hypoxanthine and guanine were focused in an unsymmetrical peak at pH 7.0 with a shoulder at pH 7.2. The activity with xanthine was focused in a symmetrical peak at pH 7.5. No activity with adenine could be detected after isoelectric focusing.

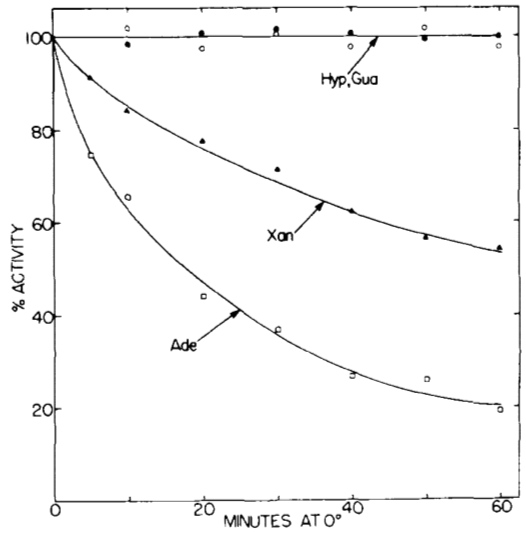

FIG. 1. **Rate of inactivation of phosphoribosyltransferase activities from *L. donovani* at 0°C.** Incubation mixtures contained 20 mM potassium phosphate, 150 mM ammonium sulfate, <0.03 mM PP-ribose-P, and 0.2 mg of protein/ml of the 40 to 80% ammonium sulfate fraction and had a final pH of 7.3. The activities with guanine (○), hypoxanthine (●), xanthine (▲), and adenine (□) at zero time were 205, 80, 43, and 105 milliunits/mg of protein, respectively.

TABLE III

Stabilizers of the purine phosphoribosyltransferase activities from L. donovani promastigotes[a]

Incubation mixtures contained 20 mM potassium phosphate, 80 mM ammonium sulfate, 0.4 mg of protein/ml of the 40 to 80% ammonium sulfate fraction, and compounds as specified below. The phosphoribosyltransferase activities with guanine, hypoxanthine, xanthine, and adenine of the unheated sample were 220, 78, 25, and 105 milliunits/mg of protein, respectively.

Additions	% activity remaining after 10 min at 37°C			
	Gua	Hyp	Xan	Ade
None	73	70	36	2.4
5 mM $MgSO_4$	97	99	34	3.5
1 mM Na_4PP-ribose-P	74	75	32	45
5 mM $MgSO_4$, 1 mM Na_4PP-ribose-P	100	100	71	102

[a] The following compounds were ineffective as stabilizers: 10 mM β-mercaptoethanol; 5 mg/ml of bovine serum albumin; 0.1 mM phenylmethylsulfonyl fluoride; 5 mM sodium pyrophosphate; and 0.1 mM NaXMP, alone and in combination with 5 mM sodium pyrophosphate.

Purification of Ade Phosphoribosyltransferase—As shown in Fig. 4, the Ade enzyme was separated from the other phosphoribosyltransferase activities by affinity chromatography on an agarose-AMP column. The activities with guanine, hypoxanthine, and xanthine were not adsorbed to agarose-AMP. The activity with adenine was eluted by the addition of 5 mM Na_4PP-ribose-P to the elution buffer. The results of a typical purification are summarized in Table IV.

The purified enzyme preparation contained no detectable contaminating enzyme activities. The lower limits of detectability expressed as a percentage of the activity with adenine were the same as for Hyp-Gua phosphoribosyltransferase from human erythrocytes (see "Experimental Procedures") except that the phosphoribosyltransferase activities with guanine, hypoxanthine, and xanthine were <0.5% and guanase was <0.03%.

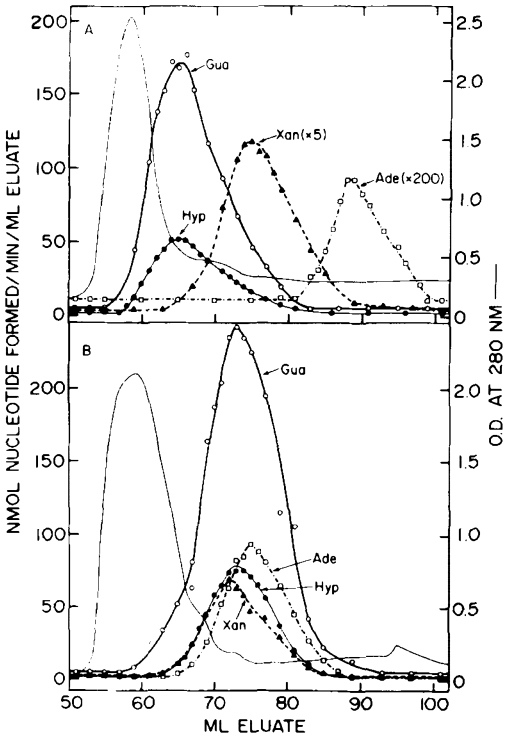

FIG. 2. **Sephadex G-100 chromatography of the phosphoribosyltransferase activities with guanine (○), hypoxanthine (●), xanthine (▲), and adenine (□) from *L. donovani* in the absence (*A*) and presence (*B*) of 1 mM PP-ribose-P.** The sample, 0.5 ml of a 40 to 80% ammonium sulfate fraction (see "Experimental Procedures") in elution buffer (see below), was applied to a column (1.5 × 90 cm). The flow rate was 10 ml/h. *A*, the column was equilibrated and eluted (4°C) with 10 mM Tris-HCl buffer, pH 7.3, containing 5 mM $MgSO_4$ and 50 mM KCl. The sample applied contained 10.7 mg of protein and 2.2, 0.83, 0.33, and 0.85 units of phosphoribosyltransferase activity with guanine, hypoxanthine, xanthine, and adenine, respectively. Activities recovered were 63, 60, 64, and 0.5%, respectively. *B*, conditions were the same as in *A* except 1 mM Na_4PP-ribose-P was present. The sample applied contained 7.6 mg of protein and 3.1, 0.91, 0.46, and 0.14 units of phosphoribosyltransferase activity with guanine, hypoxanthine, xanthine, and adenine, respectively. Activities recovered were 99, 97, 88, and 71%, respectively.

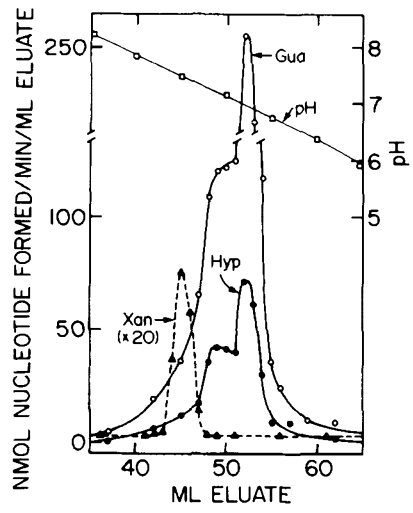

FIG. 3. **Isoelectric focusing of the purine phosphoribosyltransferase activities from *L. donovani* promastigotes.** One milliliter of a 40 to 80% ammonium sulfate fraction in 4 mM Tris-HCl buffer, pH 7.5, 1 mM $MgSO_4$, was desalted by passage down a Sephadex G-25 column (0.8 × 12 cm) equilibrated with the above buffer at 4°C. Sucrose and ampholytes were added to final concentrations of 27 and 1% to 1.7 ml of the Sephadex G-25 eluate, which contained 10.6 mg of protein/ml and 1.4, 0.49, 0.079, and 0.15 units of phosphoribosyltransferase activity with guanine, hypoxanthine, xanthine, and adenine, respectively. This sample was applied in the center of a 110-ml, 5 to 50% sucrose gradient containing 1%, pH 3.5 to 10, ampholytes in an LKB model 8100 column. A constant voltage of 600 V was applied for 36 h at 4°C. Activities recovered were 90% with guanine (○) and hypoxanthine (●), 13% with xanthine (▲), and 0% with adenine.

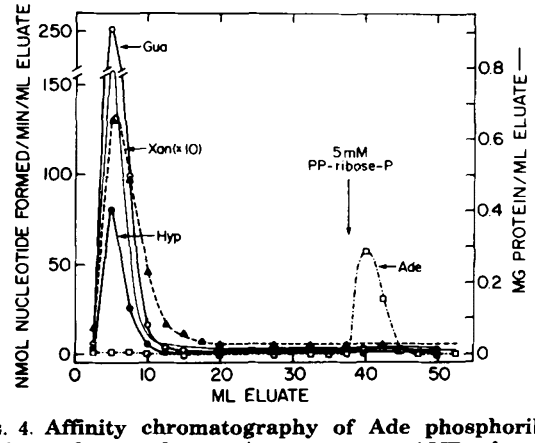

FIG. 4. **Affinity chromatography of Ade phosphoribosyltransferase from *L. donovani* on an agarose-AMP column.** The sample, 4.3 ml of a 40 to 80% ammonium sulfate fraction in 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM $MgSO_4$ and 0.1 M KCl (Buffer A), was applied to an agarose-AMP column (1 × 21 cm) equilibrated with Buffer A at 4°C. The column was eluted with Buffer A at a flow rate of 0.5 ml/min. The *arrow* indicates initiation of elution with Buffer A containing 5 mM Na_4PP-ribose-P. The sample applied contained 9.2 mg of protein and 2.1, 0.72, 0.15, and 0.90 units of phosphoribosyltransferase activity with guanine (○), hypoxanthine (●), xanthine (▲), and adenine (□), respectively. Activities recovered were 89, 88, 76, and 68%, respectively.

This enzyme preparation was stable at −70°C for at least 6 months when concentrated and stored as described for the human erythrocyte enzyme under "Experimental Procedures." In the absence of magnesium PP-ribose-P and bovine serum albumin, enzyme activity was rapidly lost at −70°C.

Purification of Hyp-Gua Phosphoribosyltransferase—The activity with hypoxanthine and guanine eluted from agarose-AMP (Fig. 4) was separated from the co-eluting Xan phosphoribosyltransferase by subsequent affinity chromatography on agarose-GTP (Fig. 5). The activity with xanthine was not adsorbed to agarose-GTP. The activities with hypoxanthine and guanine were eluted by the addition of 5 mM Na_4PP-ribose-P to the elution buffer.

The results of a typical purification are summarized in Table IV. The ratio of the activity with guanine to the activity with hypoxanthine changed only slightly during the purification of the enzyme. The purified enzyme preparation had the same stability characteristics to storage and lack of contaminating activities as noted for Ade phosphoribosyltransferase except that the phosphoribosyltransferase activity with adenine was <0.4%.

The phosphoribosyltransferase activity with xanthine, which was not adsorbed to agarose-GTP (Fig. 5), had a specific activity of 4.1 milliunits/mg of protein. This value was 5-fold lower than that of the original cell extract. This loss in specific activity is in accord with the instability of this enzyme in the cell extract, even in the presence of magnesium and PP-ribose-P (Fig. 1, Table II). Attempts to further purify the activity repeatedly resulted in low recoveries. Furthermore, the constant loss of activity of the Xan phosphoribosyltransferase

TABLE IV

Purification of Ade and Hyp-Gua phosphoribosyltransferases from L. donovani promastigotes

Fraction	Ade phosphoribosyltransferase			Hyp-Gua phosphoribosyltransferase			
	Total activity	Specific activity	Yield	Total activity[a]	Specific activity[a]	Yield	Gua/Hyp[b]
	μmol/min	*μmol/min/mg*	%	*μmol/min*	*μmol/min/mg*	%	
Cell extract	1.4	0.060	100	0.92	0.040	100	3.0
40 to 80% ammonium sulfate	0.90	0.098	64	0.72	0.078	78	3.0
Agarose-AMP eluate	0.61	2.8	44	0.63	0.093	68	3.3
0 to 80% ammonium sulfate				0.45	0.105	49	3.1
Agarose-GTP eluate				0.32	4.6	35	2.4

[a] Phosphoribosyltransferase activity with hypoxanthine.
[b] Ratio of the activity with guanine to the activity with hypoxanthine.

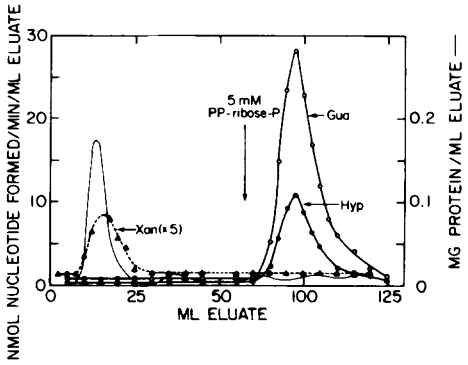

FIG. 5. **Affinity chromatography of Hyp-Gua phosphoribosyltransferase from *L. donovani* on an agarose-GTP column.** The agarose-AMP eluate (Fig. 4) which contained the phosphoribosyltransferase activities with hypoxanthine, guanine, and xanthine was brought to 80% saturation with ammonium sulfate. After stirring overnight, the suspension was centrifuged at 45,000 × *g* for 30 min and the resulting pellet dissolved in 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM $MgSO_4$ and 0.1 M KCl (Buffer A). This solution (5.4 ml) was applied to an agarose-GTP column (1 × 14 cm) equilibrated with Buffer A at 4°C. The column was eluted with Buffer A at a flow rate of 0.5 ml/min. The *arrow* indicates initiation of elution with Buffer A containing 5 mM Na_4PP-ribose-P. The sample applied contained 4.3 mg of protein and 1.4, 0.45, and 0.052 units of phosphoribosyltransferase activity with guanine (○), hypoxanthine (●), and xanthine (▲), respectively. Activities recovered were 75, 71, and 15%, respectively.

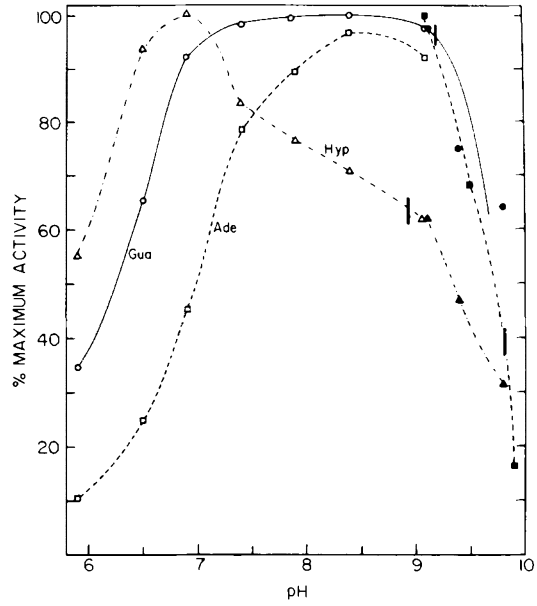

FIG. 6. **pH dependence of the rate of phosphoribosyl transfer to adenine (□) by Ade phosphoribosyltransferase and to hypoxanthine (△) and guanine (○) by Hyp-Gua phosphoribosyltransferase from *L. donovani*.** Assay conditions were as described under "Experimental Procedures" except that the buffer used was 100 mM Tris/Pipes (*open symbols*) or 100 mM Tris/Caps (*closed symbols*). The Δε values for phosphoribosyl transfer to adenine, hypoxanthine, or guanine were determined at each pH value. Maximum rates of phosphoribosyl transfer to adenine, hypoxanthine, and guanine were 3.6, 5.5, and 11 μmol/min/mg of protein, respectively. The pK_a of each purine is indicated by the position of the *solid bar*.

preparation when in solution precluded the determination of kinetic constants.

pH Optima—The effect of pH on the activities of Ade and Hyp-Gua phosphoribosyltransferases is shown in Fig. 6. Ade phosphoribosyltransferase had maximal activity at pH 8.4 to 9.1. Hyp-Gua phosphoribosyltransferase had maximal activity at pH 6.9 with hypoxanthine as substrate. With guanine as substrate this enzyme had maximal activity over a broad pH range from 7.4 to 9.1.

Specificities of Ade and Hyp-Gua Phosphoribosyltransferases from L. donovani—The apparent K_m value of magnesium PP-ribose-P was determined from double reciprocal plots of initial velocity *versus* Na_4PP-ribose-P concentration (varied between 5 and 700 μM) at a constant excess of 5 mM $MgSO_4$ (*i.e.* [$MgSO_4$] = [Na_4PP-ribose-P] + 5 mM). The apparent K_m value of magnesium-PP-ribose-P was 7 μM for Ade phosphoribosyltransferase at 50 μM adenine and 65 μM for Hyp-Gua phosphoribosyltransferase at 50 μM hypoxanthine.

Kinetic constants of some purines and purine analogues with purified Ade phosphoribosyltransferase were determined (Table V). Adenine was the most efficient substrate for this enzyme. Phosphoribosyl transfer to hypoxanthine, guanine, and xanthine was not detectable and none of these bases were appreciably inhibitory. This enzyme catalyzed phosphoribosyl transfer to purine bases having −H, $-NH_2$, or $-CH_3$, but not −OH, substituents at the 6-position. Compounds with −H, $-NH_2$, or −OH substituents at the 2-position of adenine were substrates. 4-Aminopyrazolo(3,4-*d*)pyrimidine and 8-azaadenine were 60 and 5000 times, respectively, less efficient substrates (V'_{max}/K'_m) than adenine for the enzyme. Of the nonsubstrates tested, 4-aminopyrido(2,3-*d*)pyrimidine was the best inhibitor of Ade phosphoribosyltransferase (K_i = 17 μM).

Kinetic constants for Hyp-Gua phosphoribosyltransferase purified from *L. donovani* are also listed in Table V. Apparent K_m values for guanine, hypoxanthine, and 6-mercaptopurine were similar (4 to 7 μM). The V'_{max} values for these substrates differed, phosphoribosyl transfer to guanine being the most rapid and to 6-mercaptopurine the slowest. Detectable phosphoribosyl transfer to unsubstituted purine, adenine, 6-methylpurine, 2,6-diaminopurine, or xanthine was not catalyzed by this enzyme and none of these bases were appreciably inhibitory (K_i > 1000 μM). The pyrazolo(3,4-*d*)pyrimidine analogues of hypoxanthine, 6-mercaptopurine, and guanine were 68, 26, and 33 times, respectively, less efficient substrates (V'_{max}/K'_m) than their purine counterparts. 8-Azahypoxanthine and 8-

TABLE V

Kinetic constants for Ade and Hyp-Gua phosphoribosyltransferases from L. donovani promastigotes and for Hyp-Gua phosphoribosyltransferase from human erythrocytes

Kinetic constants were determined by the direct-fit method of Wilkinson (17). Reaction mixture concentrations of Na_4PP-ribose-P and $MgSO_4$ were kept constant at 1.0 and 5.0 mM, respectively. Inhibition constants of nonsubstrates were determined from their ability to inhibit phosphoribosyltransfer by Ade phosphoribosyltransferase to 4-aminopyrazolo(3,4-*d*)pyrimidine and by Hyp-Gua phosphoribosyltransferase to 6-amino-4-hydroxyprazolo(3,4-*d*)pyrimidine. The sensitivity of the assays was increased by the use of these compounds as a result of the large $\Delta\epsilon$ values for their phosphoribosylation (Table I). A minimum of six concentrations of each substrate was used ranging from 0.17 to 0.01 mM 4-aminopyrazolo(3,4-*d*)pyrimidine and from 0.13 to 0.01 mM 6-amino-4-hydroxypyrazolo(3,4-*d*)pyrimidine.

Substrate	Ade Phosphoribosyltransferase from L. donovani				Hyp-Gua Phosphoribosyltransferase from L. donovani				Hyp-Gua Phosphoribosyltransferase from Human Erythrocytes			
	K_i[a]	K'_m	V'_{max}[b]	V'_{max}/K'_m	K_i[a]	K'_m	V'_{max}[b]	V'_{max}/K'_m	K_i[a]	K'_m	V'_{max}[b]	V'_{max}/K'_m
	(μM)	(μM)			(μM)	(μM)			(μM)	(μM)		
Purine	-	1200	30	0.025	>1000	-	<0.2(0.1)	-	-	-	-	-
6-Methylpurine	-	3700	67	0.018	>1000	-	<0.2(0.1)	-	-	-	-	-
Adenine	-	2.4	100	42	>1000	-	<0.4(0.1)	-	>600	-	<0.3(0.1)	-
2,6-Diaminopurine	-	890	20	0.022	>1000	-	<0.07(0.1)	-	-	-	-	-
6-Amino-2-hydroxy-purine	-	1400	2.2	0.0016	-	-	-	-	-	-	-	-
Hypoxanthine	>800	-	<0.2(0.1)	-	-	7.6	38	5.0	-	7.6	44	5.8
Guanine	>200	-	<0.2(0.03)	-	-	3.8	100	26	-	4.3	100	23
Xanthine	>800	-	<0.2(0.1)	-	>1000	-	<0.2(0.1)	-	>600	-	<0.8(0.1)	-
6-Mercaptopurine	-	-	-	-	-	4.5	7.1	1.6	-	8.3	20	2.4
4-Aminopyrazolo-(3,4-d)pyrimidine	-	36	23	0.64	-	-	-	-	-	-	-	-
Allopurinol	-	-	-	-	-	230	17	0.074	-	580	4.0	0.0069
6-Amino-4-hydroxy-pyrazolo(3,4-d)-pyrimidine	-	-	-	-	-	105	83	0.79	-	450	110	0.24
4-Thiopyrazolo-(3,4-d)pyrimidine	-	-	-	-	-	440	27	0.061	-	380	2.1	0.0055
7-Hydroxypyrazolo-(4,3-d)pyrimidine	-	-	-	-	20	-	<-[c](0.1)	-	3.5	-	<-[c](0.1)	-
4-Aminopyrrolo(2,3-d)-pyrimidine	950	-	<-[c](0.1)	-	-	-	-	-	-	-	-	-
4-Hydroxypyrrolo(2,3-d)-pyrimidine	-	-	-	-	340	-	<-[c](0.1)	-	-	-	-	-
8-Azaadenine	-	240	1.9	0.0079	-	-	-	-	-	-	-	-
8-Azahypoxanthine	-	-	-	-	-	6500	5.0	0.00077	-	1400	9.9	0.0071
8-Azaguanine	-	-	-	-	-	470	3.8	0.0081	-	260	18	0.069
4-Aminopyrido-(2,3-d)pyrimidine	17	-	<-[c](0.1)	-	-	-	-	-	-	-	-	-
4-Hydroxypyrido-(2,3-d)pyrimidine	-	-	-	-	>1000	-	<-[c](0.1)	-	-	-	-	-
2-Amino-4-hydroxy-pyrido(2,3-d)-pyrimidine	-	-	-	-	>100	-	<-[c](0.04)	-	-	-	-	-
4-Aminopteridine	53	-	<-[c](0.1)	-	-	-	-	-	-	-	-	-
Pterin	-	-	-	-	>100	-	<-[c](0.02)	-	-	-	-	-
5-Aminoimidazole-4-carboxamide	-	1000	8.4	0.0084	>1000	-	<0.1(0.1)	-	-	-	-	-

[a]All inhibitors showed competitive inhibition.

[b]V'_{max} values for Ade phosphoribosyltransferase are expressed as a percent of the V'_{max} of adenine (3.3 μmol AMP formed/min/mg protein), and for Hyp-Gua phosphoribosyltransferase as a percent of the V'_{max} of guanine (13 and 38 μmol GMP formed/min/mg protein for the enzymes from L. donovani and human erythrocytes, respectively). When no rate of phosphoribosyl transfer to a compound could be detected the lower limit of detectability was calculated from the amount of enzyme used and the $\Delta\epsilon$ value for the reaction. The parenthetical value after the lower limit of detectability is the mM concentration of compound used.

[c]Lower limits of detectability could not be calculated for these compounds due to the lack of $\Delta\epsilon$ values. However, when each of these compounds was incubated with purified enzyme under the assay conditions described in "Experimental Procedures", no change in absorbance between 220 and 400 nm was observed after 1 hr at 25°.

azaguanine were 6500 and 3200 times, respectively, less efficient substrates than their corresponding purines. Of the nonsubstrates tested, 7-hydroxypyrazolo(4,3-*d*)pyrimidine was the best inhibitor of this enzyme (K_i = 20 μM).

Comparison of the Specificities of Hyp-Gua Phosphoribosyltransferases from L. donovani and from Human Erythrocytes—Kinetic constants for Hyp-Gua phosphoribosyltransferase purified from human erythrocytes are also listed in Table V. The substrate efficiencies (V'_{max}/K'_m) of hypoxanthine, 6-mercaptopurine, and guanine for the enzyme from human erythrocytes were very similar to those for the enzyme from *L. donovani*. However, the pyrazolo(3,4-*d*)-pyrimidine analogues of these purines were 3 to 11 times less efficient substrates for the human enzyme than for the *L. donovani* enzyme. The converse was true for the 8-azapurine analogues of hypoxanthine and guanine, which were 9 times more efficient substrates for the human enzyme than for the *L. donovani* enzyme. 7-Hydroxypyrazolo(4,3-*d*)pyrimidine was a 6-

fold more potent inhibitor of the human enzyme than of the *L. donovani* enzyme.

DISCUSSION

L. donovani promastigotes were found to contain three distinct purine phosphoribosyltransferases: Ade, Hyp-Gua, and Xan phosphoribosyltransferases. This pattern is similar to that found in *Lactobacillus casei*, but not *E. coli* (1). The presence of a distinct Xan phosphoribosyltransferase[2] in protozoa has not been previously reported. Unlike *L. donovani*, mammalian tissues do not contain a separate Xan phosphoribosyltransferase. Human Hyp-Gua phosphoribosyltransferase has very low activity with xanthine as substrate (19, 20).

The purine phosphoribosyltransferases were present at high levels in *L. donovani* promastigotes as compared with mammalian tissues (21–24). The levels present in *L. donovani* were comparable to those found in some bacteria (1, 2).

The particle weight of the Hyp-Gua enzyme from *L. donovani* was 60,000 when determined in buffer containing magnesium and PP-ribose-P and 110,000 when determined in the same buffer lacking PP-ribose-P (Fig. 2). These values are very similar to those reported for the dimethylsuberimidate cross-linked dimer (56,000) and tetramer (108,000) of the enzyme from human erythrocytes (16). Ionic strength (25), pH, and magnesium PP-ribose-P (under some conditions) (26) have been found to affect the state of aggregation of the human enzyme. It appears that, for the *L. donovani* enzyme, incubation of the high particle weight form of the enzyme with magnesium and PP-ribose-P at pH 7.3 causes the dissociation of the enzyme into two smaller subunits. This change in particle weight under these conditions was not observed with the enzyme purified from human erythrocytes (see "Results").

Ade phosphoribosyltransferase from *L. donovani* had a particle weight of 54,000, when determined in buffer containing magnesium and PP-ribose-P, and 25,000 when determined in buffer lacking PP-ribose-P. Multiple forms of this enzyme with particle weights similar to those for the enzyme from *L. donovani* have been found in other organisms (27–29). Magnesium (27, 30), pH (30), and PP-ribose-P (28, 29) have been found to affect the state of aggregation of the enzyme from several sources.

As with the enzyme from other sources (28, 30–33), Ade phosphoribosyltransferase from *L. donovani* was partially stabilized by PP-ribose-P in the absence of magnesium. This finding is consistent with the idea that the enzyme binds PP-ribose-P in the absence of magnesium.

The pI values determined for Hyp-Gua (7.0) and Xan (7.5) phosphoribosyltransferases are considerably higher than the values reported for the enzymes from most other sources (5, 34–36). Recently, pI values of 7.85 and 8.10 have been found for Hyp-Gua phosphoribosyltransferase from beef brain (37). The shoulder on the peak of the hypoxanthine and guanine phosphoribosyltransfer activity in Fig. 3 may be due to an oligomeric form of the enzyme, or it may represent another form of the enzyme with a higher pI. Multiple forms of the enzyme having different pI values have been reported for the enzyme from other sources (34, 35, 37).

The apparent K_m value of adenine for the *L. donovani* Ade phosphoribosyltransferase (Table V) is similar to the values reported for the enzyme from many sources (5, 38–42). However, some differences in the substrate specificity of the *L. donovani* enzyme as compared with that of the mammals were found. 4-Aminopyrazolo(3,4-*d*)pyrimidine was a 7-fold more efficient substrate (V'_{max}/K'_m) and 5-aminoimidazole-4-carboxamide was a 7-fold less efficient substrate for the enzyme from *L. donovani* (Table V) as compared with the enzyme from monkey liver (38).

The apparent K_m values of hypoxanthine, guanine, and 6-mercaptopurine for the *L. donovani* Hyp-Gua phosphoribosyltransferase (Table V) are similar to the values reported for the enzyme from many sources (1, 5, 6, 35, 42–44). The rigid specificity of the *L. donovani* enzyme for substituents at the positions 6 and 2 of the purine ring (Table V) is reminiscent of the specificity of the enzyme from human erythrocytes (19). There were, however, some differences in the specificity of the parasite enzyme as compared with that of the human. Pyrazolo(3,4-*d*)pyrimidines were more efficient substrates (V'_{max}/K'_m) for Hyp-Gua phosphoribosyltransferase from *L. donovani* than for the enzyme from human erythrocytes (Table V). Conversely, 8-azapurines were less efficient substrates. The 10-fold higher substrate efficiency of allopurinol (Table V) is consistent with the observations that *L. donovani* promastigotes accumulate large quantities of allopurinol ribonucleoside 5′-monophosphate when exposed to allopurinol (10), whereas mammalian cells accumulate only minute quantities of this ribonucleotide (11). However, a 10-fold difference in substrate efficiency is probably not sufficient to account for the approximately 1000-fold difference in the accumulation of allopurinol 5′-ribonucleotide. Part of this difference may be accounted for by the 2- to 70-fold larger amount of enzyme in *L. donovani* promastigotes (Table II) as compared to mammalian cells (21–24) and by the absence of xanthine oxidase (Table II). Other factors such as PP-ribose-P levels or phosphatase activities may also contribute.

[2] There was a possibility that the Xan phosphoribosyltransfer activity in the *L. donovani* cell extracts was catalyzed by orotate phosphoribosyltransferase (EC 2.4.2.10). If so, 3-ribosylxanthine 5′-phosphate would be the expected reaction product (18). This was not the case, however. The reaction product was determined to be 9-ribosylxanthine 5′-phosphate based on its absorption spectrum and sensitivity to acid hydrolysis.

Acknowledgments—We thank Randolph Berens, Maitrayee Bhattacharyya, Kathryn Beck, and J. Joseph Marr of St. Louis University for providing the promastigotes and Donald Nelson and Gertrude Elion of the Wellcome Research Laboratories for helpful discussions.

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