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GUANOSINE 5′-MONOPHOSPHATE REDUCTASE FROM *LEISHMANIA DONOVANI*

A POSSIBLE CHEMOTHERAPEUTIC TARGET

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Abstract—GMP reductase was highly purified from promastigotes of *Leishmania donovani* by chromatography on a single DEAE-cellulose column. Bimodal substrate saturation curves resulted in a $1/v$ versus 1/[GMP] plot that curved downward above 40 μM GMP. The kinetic constants were, therefore, obtained with GMP below this concentration. The K'_m for GMP was 21 μM at pH 6.9. The enzyme was very sensitive to activation by GTP. At 20 μM GMP, a maximum of 600% activation occurred at 100 μM GTP. Half-maximal activation occurred at 8 μM GTP. GTP at 100 μM did not affect the K'_m for GMP but did increase its V'_{max} by 7-fold. Xanthosine monophosphate (XMP) and IMP analogs served equally well as competitive inhibitors versus GMP. The inhibition by the analogs and the activation by GTP were mutually antagonistic processes. The inhibition by the IMP analogs, allopurinol nucleotide and thiopurinol nucleotide is of chemotherapeutic interest because these compounds were shown previously to be produced in *Leishmania* from the anti-leishmanial agents allopurinol and thiopurinol. These nucleotides were 100- and 20-fold, respectively, more potent inhibitors of GMP reductase from *L. donovani* than of the corresponding enzyme from human erythrocytes.

It has been observed recently that allopurinol, thiopurinol and their ribonucleosides have potent anti-leishmanial activity [1–4]. An interesting biochemical feature is that these pathogenic parasites metabolically convert these compounds to substantial quantities of their corresponding nucleoside monophosphate [2–5], i.e. allopurinol ribonucleotide (HPPRP)† and thiopurinol ribonucleotide (TPPRP). Furthermore, HPPRP is uniquely converted to an AMP analog (APPRP) [2, 3, 5] via the adenylosuccinate synthetase and adenylosuccinate lyase of the parasite [7]. APPRP is further phosphorylated and incorporated into RNA [3, 5]. It is believed that the amination of HPPRP may be a lethal synthetic step for leishmania [2, 3, 5, 7]. Amination of HPPRP does not occur in mammals [8, 9] because mammalian adenylosuccinate synthetase lacks the capacity to catalyze this reaction [10].

The metabolism of TPPRP is different. It is neither aminated nor converted to the di- or triphosphate. Aside from an unidentified minor metabolite, TPPRP persists mainly unchanged [4]. Although TPPRP is a fairly good inhibitor of adenylosuccinate synthetase from *Leishmania donovani*, it also inhibits the mammalian enzyme [4] and it was not clear whether this inhibition is relevant to its anti-leishmanial activity. Furthermore, these findings raise the question as to whether the amination of HPPRP was an actual requirement for its activity. Therefore, in view of these ambiguities, it was decided to search for other enzymes that might be inhibited. The study presented here is an investigation of GMP reductase‡ from *L. donovani*.

Since GMP reductase from other sources is potently inhibited by XMP and XMP analogs with K_i values from 10^{-8} to 10^{-6} M [6, 11–13], it was possible that inhibitory, but non-detectable, levels of XMP analogs were being produced from allopurinol and thiopurinol. However, it was revealed that, compared to the inhibition of other GMP reductases, the enzyme from *L. donovani* was weakly inhibited by the XMP analogs and strongly inhibited by the IMP analogs, including HPPRP and TPPRP. Other striking differences in GMP reductases from the parasite and human sources were also established. A preliminary report of these findings has appeared elsewhere [14].

MATERIALS AND METHODS

Materials. Unless specified below, the sources of all materials may be obtained from ref. 6. [U-^{14}C]-2′-dGMP was purchased from Amersham (Arlington Heights, IL) and 6-azaUMP from the Sigma Chemical Co. (St. Louis, MO). Thiopurinol ribonucleoside monophosphate was provided by W. H. Miller of these laboratories.

Purification of nucleotides. [8-^{14}C]GMP (10 μCi) was applied onto a 0.35 × 2 cm column of Whatman DE-52 resin equilibrated in 5 mM potassium phos-

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† Abbreviations: XMP, xanthosine monophosphate; HPPRP, allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) ribonucleotide; TPPRP, thiopurinol (4-thiopyrazolo[3,4-*d*]pyrimidine) ribonucleotide; APPRP, (4-aminopyrazolo[3,4-*d*]pyrimidine) ribonucleotide. These pyrazolpyrimidines have also been referred to as 8-aza-7-deazapurine analogs [6].

‡ GMP reductase (EC 1.6.6.8) catalyzes the following reaction: GMP + NADPH + $H^+ \rightleftharpoons$ IMP + $NADP^+$ + NH_3.

phate buffer (pH 8). The column was washed with 2–3 ml of the same buffer until a small peak of radioactivity was eluted. Purified GMP was then eluted in a sharp peak by 100 mM potassium phosphate (pH 8). The radiochemical purity was found to be >99% when determined from a scan of a chromatogram that was developed as described below for the assay of GMP reductase. Samples of this material were diluted into solutions of unlabeled GMP and stored at −80°.

GTP and dGTP were purified as previously described [6].

Preparation of thin-layer plates. Cellulose MN 300 polyethyleneimine (PEI) impregnated thin-layer plates (Brinkmann Instruments, Inc., Westbury, NY) were prewashed in water by ascending chromatography. The plates were dried, and 15 μl of a mixture of 10 mM GMP and IMP carriers was applied at 2.5-cm intervals.

Enzyme assays. GMP reductase was assayed by the following procedure. Unless indicated otherwise, all reaction vials contained the following standard concentrations of reagents: 0.2 mM [^{14}C]GMP (2.5 μCi/μmole, purified), 1.9 mM NADPH, 75 mM potassium phosphate (pH 6.9), 5 mM dithiothreitol and 1 mM EDTA. The vials were pre-equilibrated to the reaction temperature of 37°, and the reactions were initiated by the addition of GMP reductase. Samples (15 μl) were withdrawn at three or more time points during the course of the reaction and were immediately applied to the PEI plates. The plates were developed by ascending chromatography for 1.5 hr in 1 M LiCl. The spots containing GMP ($R_f = 0.43$) and IMP ($R_f = 0.67$) were visualized under ultraviolet light, cut out, and placed in scintillation vials with 5 ml of Aquasol-2 (New England Nuclear Corp. Boston, MA), and the radioactivity was determined by scintillation counting. Product formation was quantitated by determining the ratio of IMP to IMP + GMP. Blank rates (enzyme omitted) had less than 0.5% of the radioactivity in the IMP spot. Velocity measurements were always within the initial, linear portion of the reactions and were also linear with the concentration of GMP reductase when measured over a range from 0 to 7 units/ml. One unit of enzyme catalyzes the formation of 1.0 nmole IMP/hr.

GMP cleaving activity was assayed under conditions that were identical to those for GMP reduction except that NADPH was omitted and the specific activity of GMP was 20 μCi/μmole. The reactions were terminated by immersion in boiling water for 3 min. Samples (3 μl) of each reaction were applied to DEAE-cellulose thin-layer plates (Brinkmann Instruments, Inc.) pre-spotted with 3 μl of 10 mM GMP. The plates were developed for 1 hr in water; this resulted in GMP remaining at the origin and all possible nucleoside or base cleavage products migrating towards the front. Each track was divided into two parts: the GMP spot (substrate), and the remaining area between this spot and the front (products). Product formation was quantitated according to the method described above for the GMP reductase assay. Blank reactions (enzyme omitted) contained less than 2% of the radioactivity in the product spot. This amount was subtracted from the complete reactions.

Purification of GMP reductase from L. donovani. Promastigotes of *L. donovani* were cultured in a semi-defined medium (HOSMEM) as described elsewhere [15]. The cells were suspended in an equal volume of 50 mM potassium phosphate (pH 7) with 5 mM dithiothreitol and stored at −80°. Approximately 5×10^{10} cells were thawed in the presence of 0.1 mM phenylmethylsulfonyl fluoride and then refrozen and rethawed two additional times. The temperature was maintained between 0 and 4° for this and all subsequent steps. The suspension was centrifuged at 49,000 *g* for 20 min, and the resultant 6.8 ml supernatant fluid was desalted on a Sephadex G-25 column equilibrated in 40 mM potassium phosphate (pH 6.9), 1 mM dithiothreitol and 1 mM EDTA. The protein pool (*ca.* 85 mg) was then applied onto a 2.6 × 7 cm DE-53 (Whatman) column equilibrated with the same buffer. The column was washed with 200 ml of this buffer at a flow rate of 8 ml/hr, and 2 ml fractions were collected. GMP reductase was then eluted in a sharp peak with 95 mM potassium phosphate (pH 6.9), 1 mM dithiothreitol and 1 mM EDTA. This enzyme peak just preceded a large protein peak that contained a combination of enzymes that rapidly converted GMP to xanthine. It was, therefore, necessary to assay for the GMP cleaving activity in order to avoid it when pooling the GMP reductase fractions. Between 500 and 900 units of GMP reductase with specific activities from 1000 to 3000 were obtained from typical preparations. The variability was dependent upon the speed by which the enzyme was stabilized by the addition of bovine serum albumin (Schwarz/Mann, Orangeburg, NY, crystallized) to 0.5 mg/ml. The enzyme was usually concentrated 3- to 4-fold with a Millipore immersible CS-10 ultra-filtration unit with no loss of activity. Aliquots (1 ml) were then fast-frozen and stored at −80°. The enzyme remained completely active for at least 3 months. The enzyme was also stable to refreezing and rethawing and extended reaction times (hours) at 37° provided it was in phosphate buffer and at least 0.1 mg/ml of bovine serum albumin.

Human GMP reductase. GMP reductase was purified from human erythrocytes as described elsewhere [6]. The enzyme was assayed in 75 mM Tris–HCl (pH 7.8), 5 mM 2-mercaptoethanol, 70 μM NADPH, 20 μM [^{14}C]GMP and various concentrations of inhibitors [6]. The reactions were analyzed as described above for the protozoal GMP reductase.

Protein assays. Protein concentrations were quantitated by the refined [16] Coomassie blue [17] method.

Kinetic constants. K_m, V_{max} and K_i values were calculated with the computer programs described by Cleland [18].

RESULTS

General properties of GMP reductase. An overwhelming GMP cleaving activity prevented the assay of GMP reductase in the crude extract. Thus, neither the increase in specific activity nor the yield of the purification could be assessed. A theoretical maximum purification of 280-fold could be calculated

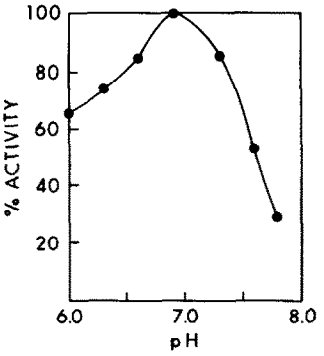


Fig. 1. The pH profile for GMP reducatse. Standard assay conditions were used with 20 μM GMP and 75 mM potassium phosphate at the indicated pH.

from the amount of protein recovered with the enzyme.

The purified enzyme preparation was found to contain maximum values of 0.6% (relative to GMP reductase) GMP cleaving activity, 6% NADPH oxidizing activity, and 8% GTP cleaving activity. These trace activities had no significant effects on the initial velocity measurements of this study. The velocity was similar for reactions containing 5 mM dithothreitol, reduced glutathione, or 2-mercaptoethanol, but it was decreased by about 50% in the absence of these sulfhydryl compounds.

The enzyme exhibited a sharp activity maximum at pH 6.9. Due to the nature of the GMP-saturation curve (see below), the profile (Fig. 1) could not be determined at saturating concentrations of GMP.

[^{14}C]2′-dGMP could be substituted for [^{14}C]GMP in the enzymic reaction. However, its rate of reduction at 0.2 mM was only 1.5% the rate observed with GMP at the same concentration.

Velocity dependence on [GMP]. The double-reciprocal graph (Fig. 2) demonstrating the relationship between the reaction velocity and the concentration of GMP showed downward curvature above 40 μM GMP. The K'_m and V'_{max} values determined for GMP within the low range (below 40 μM) were 21.2 ± 7.8 μM and 0.319 ± 0.061 nmoles per hr per unit (average of six determinations). The high range (above 40 μM GMP) V'_{max} was 6- to 8-fold faster than the extrapolated low range V'_{max}. All of the following studies were performed within the low range. Although the intracellular concentration of GMP is difficult to quantitate, it is clearly less than 40 μM.*

Activation by GTP. Purified GTP stimulated the rate of GMP reduction. When the concentration of GMP was fixed at 20 μM, optimal concentrations (100 μM) of this activator produced a maximum of 600% activation. Figure 3 demonstrates that half-maximal activation occurred at 8 μM GTP. Purified 2′-dGTP was also an activator, but produced only 32% activation at 1000 μM and 16% at 100 μM. Xanthosine produced 50% activation at 800 μM. The effect of 100 μM GTP on the K'_m and V'_{max} of GMP was assessed over the concentration range of 5 to 35 μM GMP. When compared with the values obtained in the absence of GTP, the K'_m for GMP was unchanged at 20.1 ± 3.4 μM (average of two determinations) while the V'_{max} increased 6.8-fold to 2.16 ± 0.18 nmoles per hr per unit.

Inhibition by nucleoside monophosphates. XMP, IMP, HPPRP and TPPRP were found to be strong inhibitors of GMP reductase from *L. donovani*. The inhibition was competitive with GMP. The data for the inhibition by IMP are shown in Fig. 4. Other nucleotides were also tested as inhibitors. Their K_i values were determined from Dixon plots ([GMP] fixed at 20 μM) and are presented in Table 1. For comparative purposes, the K_i values obtained with human GMP reductase are also shown in this table. To test the consistency between the Dixon method ([GMP] held constant, [I] varied) and the conventional method ([GMP] varied, [I] held constant), K_i values for XMP, IMP, and TPPRP were also determined using the Dixon method. The values obtained by the two methods varied by only 10–15%. The Dixon plot had the advantage of facilitating the

* Unpublished observation of D. J. Nelson.

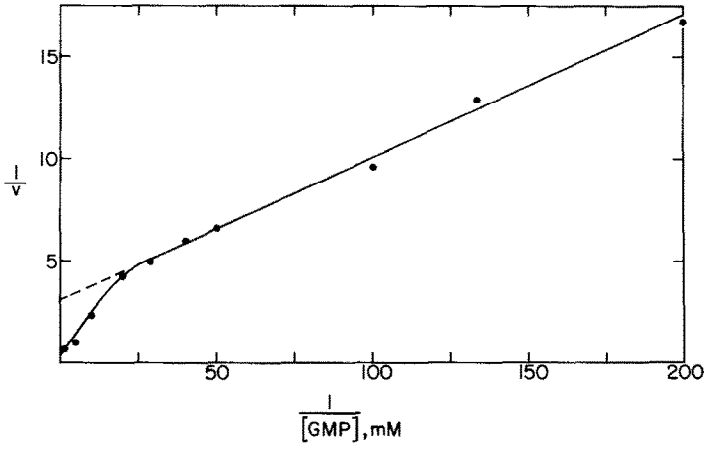


Fig. 2. Velocity dependence on the concentration of GMP. The standard assay conditions were used with [GMP] as indicated. The velocity is expressed as nmoles per hr per unit GMP reductase.

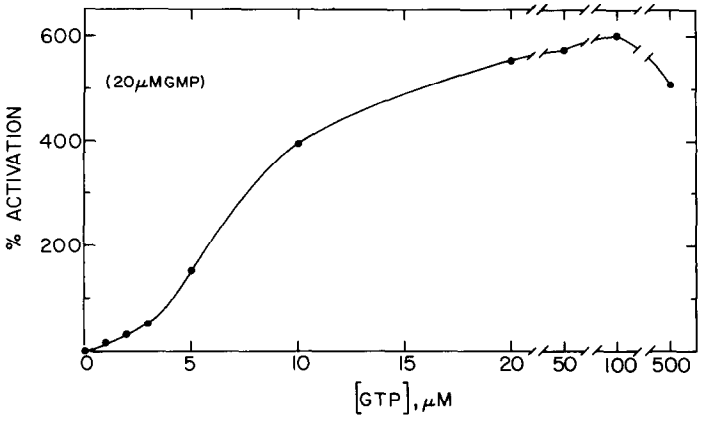


Fig. 3. Activation of GMP reductase by GTP. The standard assay conditions were used with [GMP] and [GTP] as indicated. The per cent activation equals [(activated velocity/control velocity)-1](100).

detection of deviations from linear inhibition. Such deviations were noted with a few inhibitors. AraIMP produced hyperbolic inhibition with a tangential K_i (low concentrations of araIMP) of about 15 μM and a plateau of inhibition of 75% at concentrations above 100 μM. 8-AzaGMP inhibited at concentrations below 400 μM, but activated at concentrations above 400 μM producing 25 and 42% activation at 500 and 2000 μM respectively. AcycloGMP was a weak inhibitor but produced less inhibition at 2000 μM than at 500 μM, i.e. 10 vs 24%. All other inhibitors produced linear inhibition. The following compounds had K_i values >1000 μM: cyclic XMP, OMP, adenosine, guanosine, inosine, guanine, hypoxanthine, xanthine and allopurinol riboside.

Mutual antagonism between inhibition and activation. The inhibition by the nucleotides and the activation by GTP appeared to be mutually antagonistic. This counteractive relationship is demonstrated in Fig. 5. The concentration of GTP required to achieve half-maximal activation was shifted from 8 to 25 μM in the presence of the inhibitor HPPRP (150 μM). Furthermore, the per cent inhibition by HPPRP progressively decreased as the activation by GTP increased. Dixon plots were obtained for

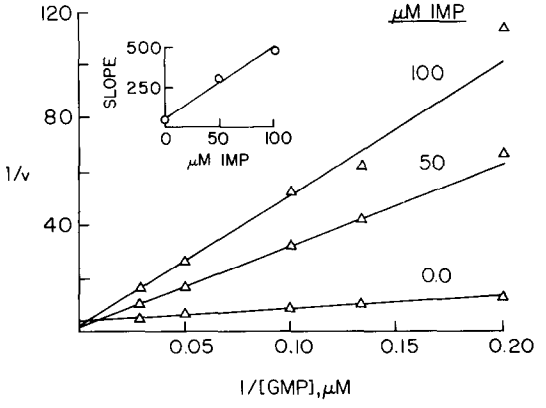


Fig. 4. Inhibition of GMP reductase by IMP. The standard assay was used with [GMP] and [IMP] as indicated. The velocity is expressed as nmoles per hr per ml GMP reductase.

HPPRP, TPPRP and XMP at 100 μM GTP ([GMP] = 20 μM). The effect of GTP was to increase significantly their apparent K_i values. The "antagonized" values were 250 μM for TPPRP and 625 μM for HPPRP. The inhibition by XMP became hyperbolic under these conditions. The tangential K_i (low concentrations) was about 15 μM and the inhibition began to plateau at 50% at 200–300 μM XMP.

DISCUSSION

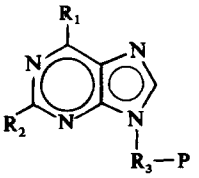
The results of this study demonstrate striking differences between the GMP reductase from this parasite and the enzyme from the human source. The differences in the catalytic properties are summarized in Table 2.

Notable differences in the inhibitor specificities of the enzymes were also observed. The finding that IMP analogs were considerably more potent inhibitors of the enzyme from *L. donovani* suggests that GMP reductase may be a potential chemotherapeutic target. Of particular interest is the inhibition of HPPRP and TPPRP, which are metabolically produced when *Leishmania* are treated with allopurinol or thiopurinol or their ribosides [2–5]. The inhibition advantage shown in Table 1 is greatly magnified by the different concentrations of these metabolities in the parasite versus the host. Treatment with their precursors results in concentrations of these nucleotides that were approximately three orders of magnitude higher in leishmanial cultures [2–5] than in mammalian tissues [8, 19]. Furthermore, since the concentrations of the inhibitors in *Leishmania* exceed their corresponding K_i values by 10- to 100-fold, selective inhibition of GMP reductase may be possible.

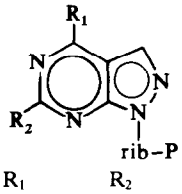
Since the human and parasite enzymes were studied at their pH optima of 7.8 and 6.9, respectively, it was possible that their K_i differences might be related to the pH differences. However, preliminary data shown here demonstrated that, at pH 6.9 with dithiothreitol replacing 2-mercaptoethanol, XMP was a very potent inhibitor and HPPRP and TPPRP were very weak inhibitors of the human GMP reductase.

It is interesting that the effects of the inhibitors

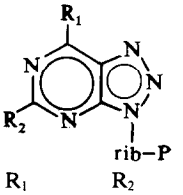
Table 1. Inhibitors of GMP reductases



Common name	R_1	R_2	R_3	Purines K_i* (μM) *L. donovani*	Purines K_i* (μM) Human†	Purines K_i* (μM) Human/*L. donovani*
XMP analogs						
XMP	O	O	Ribose	14	0.17	0.012
ThioXMP	S	O	Ribose	62	2.0	0.032
2′-dXMP	O	O	2′-Deoxyribose	*ca.* 700	27	0.039
AraXMP	O	O	Arabinose	*ca.* 360	2.5	0.007
IMP analogs						
IMP	O	H	Ribose	14	150	10.7
ThioIMP	S	H	Ribose	37	*ca.* 200	5.4
2′-dIMP	O	H	2′-Deoxyribose	*ca.* 800	*ca.* 2000	2.5
MethylthioIMP	CH_3S	H	Ribose	85		
AraIMP	O	H	Arabinose	‡	*ca.* 600	
AMP	NH_2	H	Ribose	*ca.* 250	160	0.64
GMP analogs						
ThioGMP	S	NH_2	Ribose	17	55	3.2
2′-dGMP	O	NH_2	Ribose	1300	8.2	0.006
AraGMP	O	NH_2	Arabinose	155	190	1.2
AcycloGMP	O	NH_2	CH_2—O—CH_2CH_2—O—	‡	>1000	



Common name	R_1	R_2	Pyrazolo[3,4-*d*]pyrimidines K_i* (μM) *L. donovani*	Pyrazolo[3,4-*d*]pyrimidines K_i* (μM) Human†	Pyrazolo[3,4-*d*]pyrimidines K_i* (μM) Human/*L. donovani*
XMP analogs					
1-Oxipurinol tide	O	O	57	5.0	0.088
7-Oxipurinol tide (ribose is on pyrimidine ring)	O	O	*ca.* 800	>1000	
IMP analogs					
Allopurinol tide (HPPRP)	O	H	37	3600	97
Thiopurinol tide (TPPRP)	S	H	34	680	20
AMP analog (APPRP)	NH_2	H	*ca.* 400		
GMP analog	O	NH_2	*ca.* 180	79	0.4



Common name	R_1	R_2	Triazolo[4,5-*d*]pyrimidines K_i* (μM) *L. donovani*	Triazolo[4,5-*d*]pyrimidines K_i* (μM) Human†	Triazolo[4,5-*d*]pyrimidines K_i* (μM) Human/*L. donovani*
8-AzaXMP	O	O	9.3	0.3	0.032
8-AzaIMP	O	H	9.2	*ca.* 130	14
8-AzaGMP	O	NH_2	‡	4.2	

Common name	Pyrimidines K_i* (μM) *L. donovani*	Pyrimidines K_i* (μM) Human†	Pyrimidines K_i* (μM) Human/*L. donovani*
UMP	13	>1000	>75
TMP	*ca.* 400	>1000	
CMP	*ca.* 400	>1000	
6-AzaUMP	8		

* Competitive with GMP.

† K_i values with GMP reductase from human erythrocytes were obtained from Ref. 6 for all compounds except HPPRP and TPPRP which were determined in the present study.

‡ Non-linear inhibition (see Results).

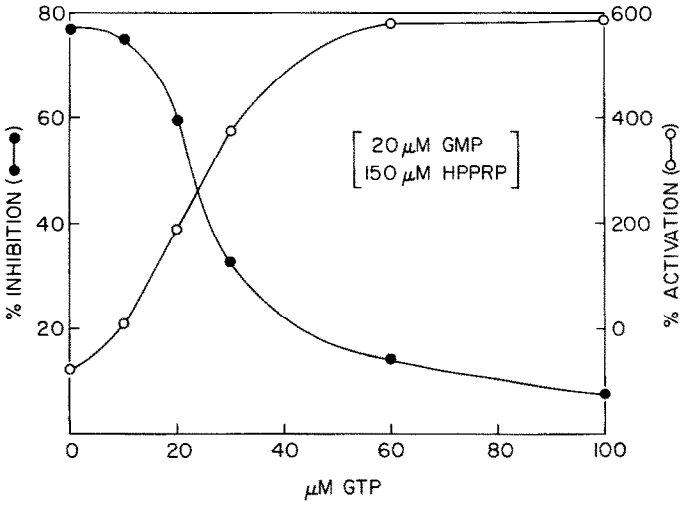


Fig. 5. Mutual antagonism between inhibition and activation. The standard assay conditions were used with [GMP], [HPPRP] (inhibitor) and [GTP] (activator) as indicated. The per cent inhibition was calculated from control reactions containing no HPPRP and the indicated [GTP]. The per cent activation was calculated from a control with no HPPRP and no GTP as described for Fig. 3. At 100 μM GTP with no inhibitor, 640% activation was observed.

Table 2. Comparison of GMP reductases from *L. donovani* and human erythrocytes

Property	*L. donovani*	Human erythrocytes*
(I) $1/v$ vs 1/[GMP]		
(A) Bimodal breaking point	40 μM	250 μM
(B) Low range K'_m	20 μM	7.5 μM
(C) V'_{max} (high range)/V'_{max} (low range)	6–8	1.4
(II) Activation by GTP		
(A) At 20 μM GMP		
(1) Maximal activation	600%	44%
(2) [GTP] for half-maximal activation	8 μM	2 μM
(B) At saturating GTP		
(1) Increase in V'_{max} for GMP	6.8-fold	1.4-fold
(III) Alternative substrate activity with dGMP		
(A) Relative velocity at 0.2 mM (GMP = 100)	1.5	1
(B) Competitive K_i (vs GMP)	1300 μM	8.2 μM

* From Ref. 6.

and the activator were mutually antagonistic. Reversal of the inhibition by XMP with GTP was observed previously with GMP reductase from *Artemia salina* [13], but neither reversal nor mutal antagonism between XMP and GTP was observed with the human enzyme [6]. Thus, these data are most consistent with the model proposed for the crustacean enzyme. That enzyme contained three putative binding sites per subunit: a substrate (catalytic) site, an activator site, and an inhibitor site [13]. The binding of an inhibitor to its site seems to be mutually exclusive with the binding of GMP to the substrate site and mutually antagonistic with the binding of GTP to the activator site. It also appears that GMP has an affinity for the activator site since at concentrations above 40 μM it incrementally increased the reaction velocity (see Fig. 2).

Although the concentrations of GTP within *L. donovani* [5] are in the range that can fully activate GMP reductase, a recent metabolic study* has revealed that GMP reductase in *L. donovani* is inhibited when these organisms are treated with either allopurinol, thiopurinol or their ribosides. It is possible that this inhibition is due to antagonism of the activation or to competition with the substrate or to a combination of these effects.

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* Unpublished data of S. W. Lafon, D. J. Nelson, R. L. Berens and J. J. Marr.

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