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# GMP REDUCTASE IN *ARTEMIA SALINA*

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## SUMMARY

1. A GMP reductase (NADPH:GMP oxidoreductase (deaminating), EC 1.6.6.8) activity has been characterized, and partially purified, in *Artemia salina* extracts. The enzyme is specific for NADPH, has a $K_m$ of 0.01 mM for GMP, is maximally active at a pH value of approx. 8.5, requires thiol groups and is resistant to heating at 55 °C for 10 min. An estimated molecular weight of 250 000 was determined by filtration on Sephadex G-200.

2. IMP has been characterized as the main product of the reaction and shown to be a competitive inhibitor ($K_i = 34\ \mu M$). The reverse reaction has been observed at 6% of the forward rate. An activation energy of 13 200 cal·mole$^{-1}$ has been found for the reaction catalyzed by this enzyme.

3. *Artemia salina* GMP reductase is not inhibited by ATP, in contrast to the enzyme previously characterized from prokaryotic organisms. The latter enzyme also has a considerably higher $K_m$ for GMP.

## INTRODUCTION

Nucleotide metabolism presents special features in *Artemia salina*. The adult female synthesizes high amounts of diguanosine tetraphosphate ($Gp_4G$) in the developing ovarium eggs [1]. The encysted eggs, which can spawn at the gastrula stage, contain about 10 $\mu$moles of $Gp_4G$ per gram (results from this laboratory). During development into free swimming larvae, they are unable to synthesize purines de novo [2], and use $Gp_4G$ as a source of both guanine and adenine nucleotides. A specific hydrolase has been described in *Artemia* extracts [3], which degrades $Gp_4G$ to GTP and GMP.

In the course of studies on the metabolic fate of GMP, we have found a GMP reductase (NADPH: GMP oxidoreductase (deaminating), EC 1.6.6.8) which catalyzes the conversion of GMP to IMP. This enzyme was described in microorganisms by Mager and Magasanik [4]. Its presence in human and rabbit erythrocytes has been inferred from the ability of these cells or hemolysates to incorporate either labelled guanine or GMP into IMP [5, 6]. However, to our knowledge, GMP reductase has not been characterized in eukaryotic cells, other than erythrocytes [5].

In this paper, the purification and properties of the GMP reductase from *A. salina*, together with a discussion on the role that this enzyme may play during de-

velopment of this crustacean are presented. A preliminary account of these findings has been published [7].

## MATERIALS AND METHODS

*Artemia* eggs from Longlife Fish Food Products, Ontario, Canada, were used in these experiments. Immediately before use, the eggs were resuspended in water and washed several times, followed by treatment with 1 % NaClO for 3 min at room temperature. After decantation, the eggs were thoroughly washed with glass-distilled water to remove residual NaClO. All further operations were performed at approx. 4 °C. The eggs were resuspended in 10 ml of ice-cold Buffer A (0.035 M Tris–HCl, pH 7.7, 0.07 M KCl, 0.009 M $MgCl_2$, 5 mM mercaptoethanol, 0.25 M sucrose) per g of original dry eggs. In a typical experiment of enzyme purification, 100 g of cysts, in 4 aliquots of 25 g each, were stirred overnight in 1000 ml of Buffer A. The homogenate was filtered through glass wool to remove cyst walls, and centrifuged at 12 000 $\times$ *g* for 15 min. The supernatant was centrifuged again at 105 000 $\times$ *g* for 75 min.

### *Assay of GMP reductase*

Unless otherwise stated, the reaction mixture contained the following components in a final volume of 1 ml: 50 mM Tris–HCl, pH 7.5, 5 mM mercaptoethanol, 0.15 mM NADPH, 0.1 mM GMP and extract in a suitable amount to give an activity of 0.005–0.01 units. Decrease in absorbance at 340 nm was followed in a spectrophotometer. Under these conditions the reaction was linear with both time and amount of extract, and was dependent on the presence of GMP in the reaction mixture (Fig. 1)

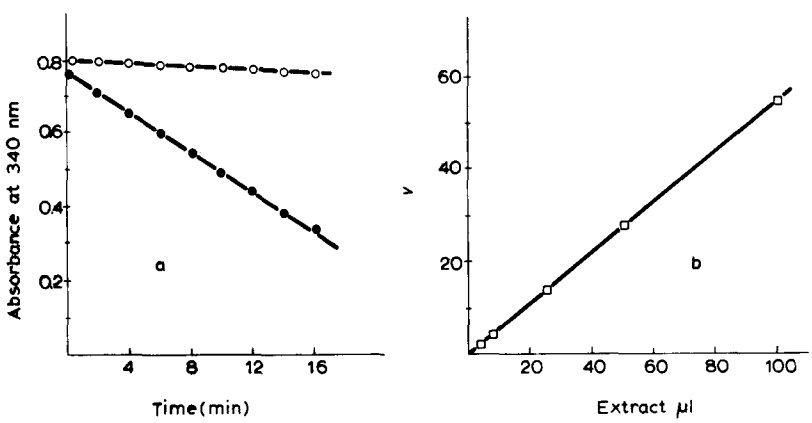


Fig. 1. GMP reductase assay in partially purified *Artemia* extracts. a, time curve; b, effect of extract concentration. Each point in B was corrected for the corresponding blank without GMP. Rest of assay conditions as described in the text. ●—●, complete system; ○—○, without GMP.

### *Other materials*

Nucleotides were purchased from Boehringer Mannheim and Sigma. [$^{14}$C]-GMP (520 mCi/mmole) from the Radiochemical Centre, Amersham. Glucose oxidase

and fibrinogen bovine Fraction I, Type I, were from Sigma. Other enzymes used were from Boehringer. Fibrinogen was purified before use by the method of Laki [8].

## RESULTS

### *Purification*

All operations were carried out at approx. 4 °C. The starting material was the 105 000 × *g* supernatant obtained as described in Material and Methods.

*Step 1, $(NH_4)_2SO_4$ fractionation.* The 105 000 × *g* supernatant, in a volume of 785 ml was brought to 45% saturation with $(NH_4)_2SO_4$, stirred for 60 min and centrifuged at 27 000 × *g* for 15 min. The precipitate was discarded and the supernatant brought to 70% saturation with $(NH_4)_2SO_4$ and treated as above. The precipitate was resuspended in Buffer B (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 0.5 mM EDTA), and dialyzed against 2 l of Buffer B for 2 h followed by an overnight dialysis against 2 l of the same buffer.

*Step 2, heating.* The dialyzed solution from the previous step (about 36 ml) was placed in a water bath at 55 °C and continuously stirred. After reaching 55 °C, the solution was kept in the water bath for 10 min. After quickly cooling in an ice bath, the mixture was centrifuged at 12 000 × *g* for 15 min and the precipitate discarded.

*Step 3, chromatography on Sephadex G-200.* The supernatant from Step 2 (26.5 ml) was applied to a Sephadex G-200 column (91 cm × 2.6 cm) equilibrated with Buffer B and eluted with the same buffer. Fractions of 8.3 ml were collected. Fractions 25–32 containing the major portion of GMP reductase activity were collected (Fig. 2).

*Step 4, chromatography on DEAE-cellulose.* The pooled fractions from the pre-

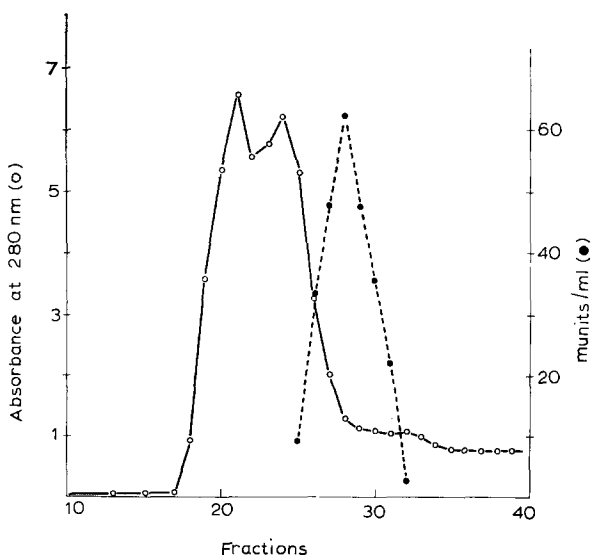


Fig. 2. Gel filtration of the 45–70% $(NH_4)_2SO_4$ fraction on Sephadex G-200. Step 3 of purification as described in the text.

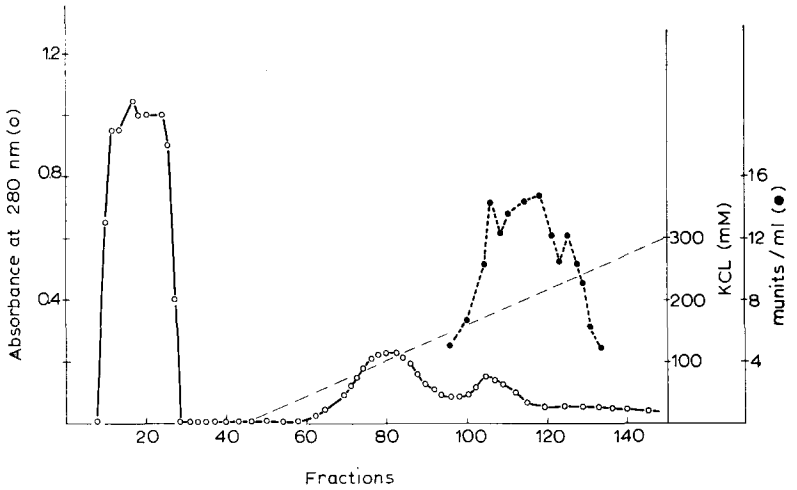


Fig. 3. Purification of GMP reductase by chromatography on DEAE-cellulose. The pooled fractions from the previous step (Fig. 2), containing the major portion of the GMP reductase activity were applied to a DEAE-cellulose column and after washing with buffer B, GMP reductase was eluted with a linear gradient of KCl. Details as described in the text (Step 4).

vious step (62 ml) were applied to a column (18 cm × 1.5 cm) of DEAE-cellulose previously equilibrated in Buffer B (Fig. 3). The column was washed with 180 ml of the same buffer. GMP reductase was then eluted with 450 ml of a linear gradient (0.05–0.3 M) of KCl in Buffer B. Fractions of 4.1 ml were collected. GMP reductase was eluted between Fractions 98 and 132 (0.18–0.26 M KCl). The solution containing the enzymatic activity (142 ml) was concentrated by precipitation with $(NH_4)_2SO_4$ (90% saturation). The precipitate was resuspended in 2 ml of Buffer B and dialyzed against the same buffer to remove residual $(NH_4)_2SO_4$.

A summary of a typical purification run is given in Table I. To assay GMP reductase in the 105 000 × *g* supernatant, an aliquot (1 ml) was dialyzed against 2 l of Buffer B for 2 h followed by an overnight dialysis against 2 l of the same buffer. As seen in Table I, a purification of 100-fold with a recovery of 35% was obtained.

TABLE I

PURIFICATION OF *A. SALINA* GMP REDUCTASE

100 g of cysts were used. One unit is the amount of enzyme able to transform 1 μmole of GMP per min at 30 °C. Proteins were measured by the method of Lowry et al. [9].

| Step | Activity (units) | Protein (mg) | Specific activity (munits/mg) | Recovery (%) | Purification factor |
|---|---|---|---|---|---|
| Supernatant 105 000 × *g* | 3.68 | 4 710 | 0.8 | 100 | |
| 45–70% $(NH_4)_2SO_4$ | 3.24 | 2 200 | 1.5 | 88 | 2 |
| Heated at 55 °C | 2.67 | 932 | 2.9 | 72 | 4 |
| Sephadex G-200 | 2.48 | 137 | 18.2 | 67 | 23 |
| DEAE-cellulose | 1.27 | 16 | 78.0 | 35 | 100 |

All the experiments described below were carried out with the concentrated material obtained from DEAE-cellulose.

*Properties of GMP reductase*

*Michaelis constant for GMP.* Under the assay conditions described in Materials and Methods, typical hyperbolic kinetics was observed for this reaction. From a Lineweaver–Burk plot a $K_m$ of 0.01 mM was calculated (Fig. 4, open circles).

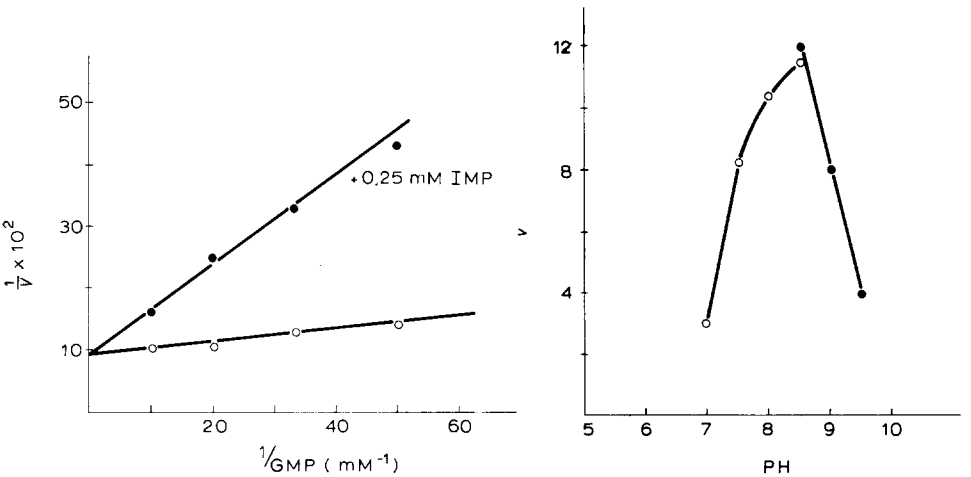


Fig. 4. Effect of GMP concentration, in the presence or absence of IMP, on the initial velocity of GMP reductase. Conditions were as described in the text, except for GMP and IMP concentrations as indicated.

Fig. 5. Influence of pH on the GMP reductase activity. An enzyme preparation from DEAE-cellulose was assayed at different pH values using 50 mM Tris–Maleate buffer (pH 7–8.5) (open circles) and 50 mM Glycine, NaCl–NaOH buffer (pH 8.5–9.5) (filled circles).

*Influence of pH and temperature.* As can be seen in Fig. 5, the optimum pH is approx. 8.5. The experiment was carried out using 50 mM Tris–maleate buffer and 50 mM glycine, NaCl–NaOH buffer. The point corresponding to pH 8.5 was obtained with the two buffers. No buffer effect on the velocity of the reaction was observed in this case.

From an Arrhenius plot, an activation energy of 13 200 cal·mole$^{-1}$ was obtained for this reaction.

*Substrates, stoichiometry, and apparent irreversibility of the reaction.* The enzyme is specific for NADPH. When the reaction was carried out with NADH (0.15 mM) in the standard assay conditions, the actual velocity was less than 2% of that obtained with NADPH. 1 mole of NADPH was oxidized per mole of GMP consumed (results not shown).

When the reaction was carried out with labelled GMP and the products of the reaction separated by paper electrophoresis, the radioactivity initially present as GMP was progressively displaced in the course of incubation to a compound which had the same electrophoretic mobility as the IMP used as marker (Table II). At each time of incubation the ratio between the cpm which had disappeared from GMP

TABLE II

STOICHIOMETRY OF GMP REDUCTASE REACTION

The reaction mixture for the assay of GMP reductase contained the following components in a final volume of 0.1 ml: 50 mM Tris–HCl, pH 7.5, 5 mM mercaptoethanol, 0.2 mM NADPH, 0.2 mM [U-$^{14}$C]GMP (19.2 $\mu$Ci/$\mu$mole) and 0.0025 units of an enzyme preparation from DEAE-cellulose. The reaction was carried out at 30 °C. Aliquots of 10 $\mu$l were taken at zero time and 5, 10, 15 and 20 min after the reaction was started. In each case the reaction was stopped by heating at 90 °C for 1 min. 5-$\mu$l portions of each of the above aliquots were placed on a Whatman No. 1 paper which had been previously spotted with a mixture of non-labelled GMP and IMP (0.5 $A_{260\ nm}$ units each) in order to locate thereafter the nucleotides by ultraviolet light. The paper electrophoresis was run for 3 h at 30 V/cm in 20 mM citrate buffer, pH 3.5. The spots corresponding to GMP and IMP were cut out and the radioactivity counted in a Nuclear Chicago Mark II Scintillation Counter with Permablend™ I solution as scintillation solvent. A corresponding aliquot of the reaction mixture spotted on a piece of Whatman No. 1 paper gave the same radioactivity as the spot corresponding to GMP (zero time) or the sum of the radioactivity under the spots corresponding to GMP and IMP, thus showing that all the radioactivity in the assay mixture is recovered after electrophoresis as IMP and/or GMP. Under the conditions in which the radioactivity was counted, $3.2 \cdot 10^4$ cpm correspond to 1 nmole of chemical compound. For additional details see the text.

| Incubation time (min) | GMP (cpm) | IMP (cpm) | $\Delta$GMP/$\Delta$IMP |
|---|---|---|---|
| 0 | 31 058 | 200 | – |
| 5 | 24 803 | 4 964 | 1.3 |
| 10 | 17 820 | 12 522 | 1.08 |
| 15 | 15 373 | 17 384 | 0.91 |
| 20 | 13 246 | 17 599 | 1.02 |

and those recovered as IMP was approx. 1 (Table II), hence for every molecule of GMP reduced, 1 molecule of IMP was formed.

In order to assess the identity of IMP as the product of the reaction, GMP reductase was incubated at 30 °C in a reaction mixture containing the following components in a final volume of 0.2 ml: 50 mM Tris–HCl, pH 7.5, 5 mM mercaptoethanol, 10 mM GMP, 10 mM NADPH and 25 mU of enzyme. The reaction was stopped as described in Table II. At 0 and 40 min of incubation, 20-$\mu$l aliquots were spotted on a Whatman 3MM paper and subjected to electrophoresis in 20 mM citrate buffer, pH 5.0, for 3 h at 30 V/cm. In the case of the 40-min sample a new spot appeared at the ultraviolet light region, which was not present in the 0-min sample. This new spot, with the same mobility as a control of IMP, was eluted and characterized as IMP by its ultraviolet-absorption spectrum ($\lambda_{max} = 249$, $A_{280\ nm}/A_{260\ nm} = 0.26$, $A_{250\ nm}/A_{260\ nm} = 1.48$, at pH 2.0; $\lambda_{max} = 253$, $A_{280\ nm}/A_{260\ nm} = 0.20$, $A_{250\ nm}/A_{260\ nm} = 1.12$, at pH 11).

The reversibility of the reaction was investigated following the reduction of $NADP^+$ (0.3 mM) in the presence of 1 mM IMP and 1 mM $NH_4Cl$; the remainder of the assay mixture was as described under Materials and Methods. Under these conditions, the velocity of the reverse reaction was 6% of that in the forward direction.

*Requirement of thiol groups.* The enzyme requires the presence of thiol groups in the reaction mixture to be fully active. In the absence of mercaptoethanol, 50% of the maximal velocity was observed. The residual activity could be completely abolished by 35 $\mu$M *p*-hydroxymercuribenzoate. The effect of this reagent, at this concentration, can be overcome with 5 mM mercaptoethanol.

*Effect of nucleotides.* The reaction mixture for the study of the effect of nucleotides on the GMP reductase contained 5 mM $MgCl_2$ in addition to the usual components. The reaction was followed in the presence of 0.1 mM GMP and at 1 and 2 mM nucleotides concentration. No effect on the velocity of the reaction was observed with ATP, GTP, ITP, UTP, ADP, GDP and UMP. With AMP, 10% and 20% inhibition was observed at 1 and 2 mM concentration, respectively. An inhibition of 76% occurred at 1 mM IMP. As shown in Fig. 4, this inhibition is competitive in relation to the GMP concentration, thus showing it to be an inhibition byproduct. A $K_i$ of 0.035 mM for IMP was calculated (Fig. 4).

The kinetic experiments performed by Mager and Magasanik [4], were carried out without $Mg^{2+}$ in the reaction mixture for the assay of GMP reductase (from microorganisms). In these conditions, an inhibition of 95% was observed with ATP (1 mM) when a concentration of 1 mM GMP was used [4]. We also assayed ATP as effector at 1 mM concentration, without $Mg^{2+}$ in the reaction mixture, and at 0.1 mM GMP. In contrast to the bacterial GMP reductase, no inhibition by ATP was observed with the *Artemia* enzyme.

*Molecular weight*

The molecular weight of GMP reductase was determined by gel filtration using a Sephadex G-200 (Pharmacia) column, previously equilibrated with Buffer B. The column size was 1.5 cm × 93 cm (bed volume 164 ml). The sample applied to the column contained the following components in a final volume of 1.57 ml of Buffer B: GMP reductase (28 mg of protein, 0.08 units), and the following markers: yeast hexokinase (0.25 mg, 35 units); rabbit muscle glyceraldehyde-3-phosphate dehygenase (0.3 mg, 10 units); *Aspergillus niger* glucose oxidase (0.07 mg, 14 units); rabbit muscle pyruvate kinase (0.1 mg, 15 units), and bovine fibrinogen (3 $A_{280\ nm}$

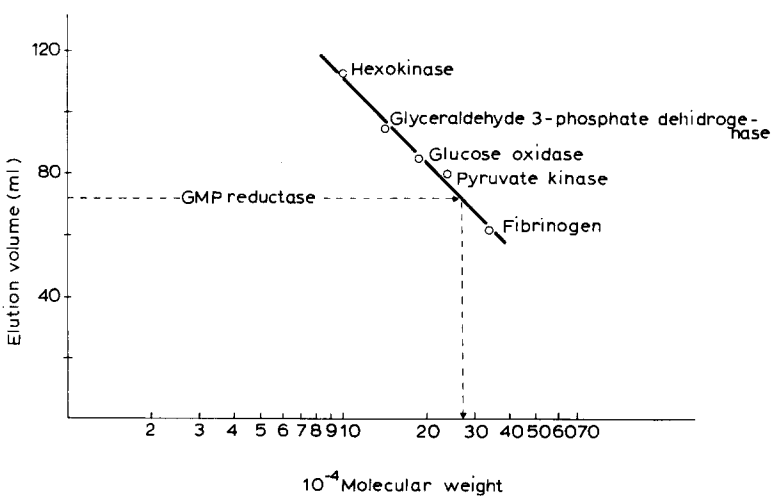


Fig. 6. Determination of estimated molecular weight of GMP reductase by Sephadex G-200 gel filtration. The column was calibrated with proteins of known molecular weight as described in the text. An enzyme preparation from Step 3 (heating) was used in this experiment. GMP reductase activity was eluted as a sharp symetric peak ($V_e$ = 72 ml) corresponding to a molecular weight of 265 000.

units). Column effluents were collected in 1.06-ml fractions, at a flow rate of 10 ml/h. Reference enzymes were estimated by suitable assays, and fibrinogen by absorbance at 280 nm. The effluent volume corresponding to the maximal enzyme activity or protein concentration (elution volume, $V_e$) was determined to the nearest ml from the elution profile by extrapolating both sides of the activity or protein peak to an apex. An estimated molecular weight of 265 000 was calculated for GMP reductase (Fig. 6).

## DISCUSSION

*A. salina* GMP reductase is strictly specific for NADPH (as is the case with that from microorganisms [4]), is maximally active around pH 8.5 (Fig. 5), is resistant to heating at 55 °C for 10 min (Table I), requires thiol groups and has no requirement for $Mg^{2+}$. In contrast, the two enzymes have different $K_m$ values for GMP, 0.01 mM and 0.1 mM for the enzyme from *Artemia* and microorganisms, respectively, and behave differently in relation to the inhibition by ATP. With the enzyme from microorganisms, an inhibition of 95% was obtained with 1 mM ATP at 1 mM concentration of GMP [4]. In our hands, no inhibition was observed by ATP (2 mM) assaying the *Artemia* enzyme at 0.1 mM GMP (see Results).

As outlined in the introduction, *A. salina* embryos use $Gp_4G$ as a source of purine nucleotides during development. A specific hydrolase cleaves this compound to GTP and GMP [3]. In the course of studies on the metabolic fate of GMP, the reductase activity described in this paper was found in *Artemia* extracts. This enzymatic activity could imply a shuttle in the pathway from GMP to AMP. The similarities in the $K_m$ (around 10 $\mu$M) and $V$ (around 80 mU/g) of the diguanosine tetraphosphatase (EC 3.6.1.17) (manuscript in preparation), and the GMP reductase towards their respective substrates, make it probable that both enzymes are acting in sequence in vivo to convert the GMP moiety of $Gp_4G$ into IMP. Nevertheless, to elucidate whether GMP is metabolized preferentially by the action of the GMP reductase, an evaluation of the $V$ and $K_m$ of the enzymes acting at the GMP crossroads would be desirable.

## NOTE ADDED IN PROOF (Received February 4th, 1974)

GMP reductase has been very recently described in calf thymus and found to be strongly inhibited by XMP. [10].

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