Biochemical Pharmacology, Vol. 18, pp. 2747–2757. Pergamon Press 1969. Printed in Great Britain

# INHIBITION OF INOSINIC ACID DEHYDROGENASE OF SARCOMA 180 ASCITES CELLS BY NUCLEOTIDES AND THEIR ANALOGS*

JULIUS H. ANDERSON† and ALAN C. SARTORELLI

Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. 06510, U.S.A.

(*Received* 12 *February* 1969; *accepted* 16 *May* 1969)

**Abstract**—IMP dehydrogenase of sarcoma 180 ascites tumor cells is inhibited in a competitive manner by the guanine nucleotide biosynthetic pathway end-product GMP. The effectiveness of IMP as a substrate and of GMP as an inhibitor was mimicked less strongly by dIMP and dGMP, as well as by the corresponding nucleoside di- and triphosphate compounds. 8-Azaguanosine 5′-phosphate, 2-amino-6-chloropurine ribonucleoside 5′-phosphate, 2,6-diaminopurine ribonucleoside 5′-phosphate and AMP, which also may be considered to be derivatives of GMP and IMP, produced inhibition of enzymatic activity. IMP, XMP and GMP behaved similarly in protecting IMP dehydrogenase from inhibition by *N*-ethylmaleimide. No evidence was found requiring the postulation of an allosteric site in the mammalian enzyme for the feedback inhibitor GMP.

INOSINIC acid dehydrogenase (IMP dehydrogenase; IMP : $NAD^+$ oxidoreductase, EC 1.2.1.14) catalyzes the following reaction:

$$IMP + NAD^+ + H_2O \longrightarrow XMP + NADH + H^+.$$

This enzymic step occurs immediately after a branch point in the purine nucleotide biosynthetic pathway; thus, IMP dehydrogenase may be considered to be the first enzyme uniquely involved in the biosynthesis of guanine nucleotides. As such, it is a potential locus of control of guanine nucleotide and nucleic acid biosynthesis by intrinsic cellular metabolic control mechanisms and is, therefore, also a prime target for metabolic attack by pharmacological agents that mimic physiologic controls.

Mager and Magasanik[1] demonstrated inhibition *in vitro* of the activity of IMP dehydrogenase of *Aerobacter aerogenes* by the pathway end-products, GMP and GDP. Hampton and Nomura[2] investigated the kinetic aspects of the inhibition of this enzyme by GMP and showed that the inhibition is classically competitive with respect to the substrate, IMP. In contrast, Buzzee and Levin[3] working with partially purified cell-free extracts of *Salmonella typhimurium* and Ishii and Shiio[4] employing an IMP dehydrogenase preparation from *Bacillus subtilis* reported nonlinearities in the inhibition produced by GMP in Lineweaver–Burk double reciprocal plots of substrate

* This work was presented to the Graduate School of Yale University by J. H. Anderson in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Support was provided by United States Public Health Service Grant CA-02817 and Training Grant 5-T1-GM59, and an American Cancer Society Postdoctoral Research Scholarship to J. H. A.

† Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104.

(IMP) concentration and initial velocities and suggested that these deviations from linearity might be produced by allosteric phenomena. In support of this concept a difference in the effects of alterations in pH on the apparent $K_m$ for IMP and the apparent $K_i$ for GMP were demonstrated;[3] these findings were attributed to different ionizing groups at separate binding sites for IMP and the negative effector GMP.

Since differences exist in the mechanism by which guanine nucleotides interact with IMP dehydrogenase enzymes from microbial cells, and no data are available on the feedback inhibition by GMP of IMP dehydrogenase of mammalian cell origin, the present report describes the specificity and mechanism of interaction of IMP dehydrogenase of sarcoma 180 ascites cells with various nucleotides and their analogs. Some of these data have appeared previously in abstract form.[5,6]

## MATERIALS AND METHODS

*Enzyme preparation.* IMP dehydrogenase activity was isolated from 6-day growths of sarcoma 180 ascites cells as detailed earlier.[7] The procedure consisted of sonication of neoplastic cells, centrifugation at 105,000 *g* for 2 hr, and fractionation of the supernatant solution with ammonium sulfate. The precipitate which formed between 20 and 40% saturation with ammonium sulfate was collected, dialyzed against 0·01 M Tris-Cl (pH 8) and stored frozen at − 17° for about 1 week; gradual loss in enzymatic activity occurred after longer periods of storage. This preparation contained about 4% of the total cellular protein, did not significantly destroy IMP, $NAD^+$, or NADH, and required IMP, $NAD^+$ and an inorganic cation for activity.

*Enzyme assay.* The activity of IMP dehydrogenase was monitored by the method of Magasanik *et al.*,[8] which is based upon the determination of the increase in absorbancy at 340 mμ, a measure of the net production of NADH. Assays were routinely conducted in the following manner: 0·1 M Tris-Cl (pH 8), 0·1 M KCl, 0·28 mM $NAD^+$ ($K^+$ salt), 0·1 ml of enzyme preparation (generally 1 to 3 mg of protein), inhibitor where indicated, and water to give a volume of 0·9 ml were mixed by inversion in a 1-ml quartz cuvette. The cuvettes were allowed to equilibrate to 37°, the temperature of the cell chamber being maintained at 37° ± 1° with a Tanford thermostated circulating water bath. After 5 min of preincubation, 2·3 mM IMP ($Na^+$ salt) in a volume of 0·1 ml, prewarmed to 37°, was added to initiate the reaction. The change in absorbancy at 340 mμ was a linear function of the amount of enzyme used in the reaction. Lines were fitted to the data in the double reciprocal plots by use of an IBM computer with the program of Cleland.[9]

*Chemicals.* IDP and ITP were obtained from Fluka A.G., Switzerland, and were purified by ion-exchange chromatography on a column of DEAE-cellulose using a triethylammonium bicarbonate gradient from 0·005 to 0·5 M. IMP, $NAD^+$, NADH, GMP, GDP, GTP, dGMP, AMP, dAMP, UMP and 8-azaguanosine 5′-phosphate (8-azaGMP) were obtained from P-L Biochemicals, Inc. 6-Chloropurine ribonucleoside was purchased from Sigma Chemical Co. 2,6-Diaminopurine ribonucleoside and 2-amino-6-chloropurine ribonucleoside were supplied by Cyclo Chemical Co.; the latter compound was also obtained as a gift from Dr. Roland K. Robins. *N*-Ethylmaleimide was purchased from CalBiochem.

*Synthetic methods.* The thin-layer ion-exchange chromatographic method of Randerath and Randerath[10] and paper chromatography (Whatman No. 1 or Whatman No. 3MM) using a descending flow of solvent were employed in the synthesis and/or

purification of compounds. The solvent systems used in paper chromatography were (a) absolute alcohol: 1 M ammonium acetate (pH 7·4), 7 : 3 (v/v)[11]; (b) 0·1 M potassium phosphate buffer (pH 6·4);[12] (c) 1 % $(NH_4)_2SO_4$ : isopropyl alcohol, 1 : 1 (v/v);[13] and (d) isopropyl alcohol : $NH_4OH$ : water, 14 : 1 : 15 (v/v/v).[14]

The 2′, 3′ isopropylidene derivative of 2, 6-diaminopurine ribonucleoside was prepared by the method of Hampton and Magrath.[11] This compound was converted to the 5′-ribonucleotide by the method of Roy *et al.*[15] The $R_f$ value of the product (0·3) in solvent system a corresponded to that obtained by Way and Parks[16] using 2, 6-diaminopurine ribonucleoside 5′-phosphate (DAMP) prepared enzymatically. On thin-layer ion-exchange chromatography a single spot was obtained ($R_f$ 0·38). The spectral properties of the product were identical to those of the starting material.

6-Chloropurine ribonucleoside 5′-phosphate (ClIMP) was synthesized and purified by the method of Hampton and Maguire.[17] A single spot with $R_f$ 0·31 was obtained using the thin-layer ion-exchange chromatographic system.

The 2′, 3′-isopropylidene derivative of 2-amino-6-chloropurine ribonucleoside was prepared as described for other compounds by Hampton and Magrath,[11] the insertion of the isopropylidene group being associated with a change in $R_f$ from 0·75 to 0·85 in solvent system c. Phosphorylation of the 5′-position with pyridinium cyanoethylphosphate in the presence of dicyclohexylcarbodiimide was accomplished by the method of Roy *et al.*;[15] this reaction was detected by a change in $R_f$ from 0·59 to 0·76 in solvent system b. The isopropylidene group was then removed as described by Roy *et al.*[15] The cyanoethyl group was removed by adjustment of the solution to pH 12 with NaOH, and incubation for 10 min at 50°; this treatment changed the $R_f$ of the compound from 0·83 to 0·35 in solvent system d. The product was purified by chromatography on a 4·5 × 33 cm column of DEAE-cellulose using a 0·005 to 0·3 M nonlinear gradient of triethylammonium bicarbonate (compound eluted after approximately 1·2 l.) and by subsequent chromatography on paper with solvent system a. The final product (2-amino-6-chloropurine ribonucleoside 5′-phosphate) chromatographed in solvent system a ($R_f$ 0·38) similarly to other nucleotides (IMP $R_f$ 0·37, GMP $R_f$ 0·40) and differently from ribonucleosides (6-chloroguanosine $R_f$ 0·83–0·90, guanosine $R_f$ 0·71) and purine bases (6-chloroguanine $R_f$ 0·58–0·67, 2, 6-diaminopurine $R_f$ 0·66). In chromatography on the thin-layer ion-exchange system, the reaction product again behaved similarly to other purine mononucleotides (ClGMP $R_f$ 0·27, IMP $R_f$ 0·33, GMP $R_f$ 0·29). The product had an ultraviolet absorption peak at 310 mμ in acid and at 305 mμ in neutral and alkaline conditions with a minimum at 265 mμ at all pH values; these spectral properties were similar to those of the starting material. The overall yield was 5 mg of the ammonium salt of the nucleotide from 500 mg of the ribonucleoside.

Prior to use the synthetic nucleotides were converted to potassium salts by stirring with Dowex 50-$K^+$ in water.

## RESULTS

The inhibition of IMP dehydrogenase of sarcoma 180 cells by GMP was competitive with IMP (Fig. 1). In contrast to findings in microbial systems,[3,4] the data obtained with the mammalian enzyme were linear when plotted in the double reciprocal form of Lineweaver and Burk. The effect of GMP on the slope of the lines was a linear function of the concentration of GMP. The apparent $K_i$ for this experiment was $7{\cdot}5 \times 10^{-4}$ M;

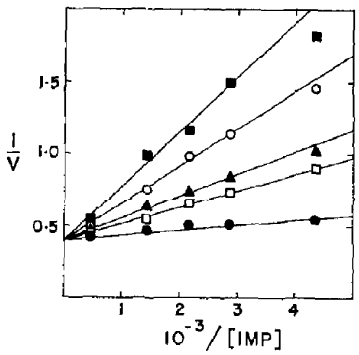


FIG. 1. Double reciprocal plot of the inhibition of IMP dehydrogenase by GMP. Where indicated GMP was added to the preincubation mixture. The data shown are from one of several experiments which yielded similar results. V is initial velocity in mμmoles per min. Brackets indicate molar concentration. ●, No GMP; □, $1{\cdot}2 \times 10^{-3}$ M GMP; ▲, $2{\cdot}4 \times 10^{-3}$ M GMP; ○, $4{\cdot}7 \times 10^{-3}$ M GMP; ■, $7{\cdot}0 \times 10^{-3}$ M GMP.

TABLE 1. EFFECT OF SEVERAL NUCLEOTIDES ON THE ACTIVITY OF IMP DEHYDROGENASE*

| Inhibitor | Concn of inhibitor (mM) | Concn of IMP (mM) | Percentage inhibition |
|---|---|---|---|
| GMP | 1·2 | 0·023 | 84 |
| | | 0·23 | 42 |
| GDP | 4·5 | 0·023 | 5 |
| | | 0·23 | (7)† |
| | | 2·3 | (21)† |
| GTP | 3·2 | 0·023 | 31 |
| | | 0·23 | 9 |
| IDP | 1·8 | 0·23 | 19 |
| ITP | 0·87 | 0·23 | 27 |
| dGMP | 1·7 | 0·023 | 30 |
| | 3·4 | 0·023 | 55 |
| | | 0·23 | 12 |
| dAMP | 1·3 | 0·023 | 10 |
| | 2·7 | 0·023 | 34 |
| UMP | 6·2 | 0·023 | 62 |
| | | 0·23 | 29 |

* The sodium salts of the indicated nucleotides were preincubated with the enzyme preparation for 5 min before addition of substrates. The percentage inhibition was determined relative to the activity of the enzyme at the indicated concentrations of IMP. The concentration of $NAD^+$ was $2{\cdot}8 \times 10^{-4}$ M in all experiments.

† Stimulation.

in other experiments the $K_i$ ranged from 2 to 8 $\times$ $10^{-4}$M. The apparent $K_m$ for IMP was 2 $\times$ $10^{-5}$ M. With $NAD^+$ as the variable substrate, GMP produced non-competitive inhibition.

To investigate the structural specificity of the nucleotide for inhibition of IMP dehydrogenase, several nucleotides were examined for inhibitory activity. Inclusion of additional phosphate groups on the guanylate molecule lowered the degree of inhibition (Table 1). Thus, GDP at a concentration of 4·5 $\times$ $10^{-3}$ M was not inhibitory and GTP at 3·2 $\times$ $10^{-3}$ M produced significant inhibition only at relatively low concentrations of IMP, IDP and ITP at relatively high concentrations appeared to partially substitute for IMP as substrates for IMP dehydrogenase. Since partial breakdown of these compounds to IMP could not be eliminated, IDP and ITP were at best poor substrates for the enzyme. On the other hand, IDP and ITP appeared to be relatively good inhibitors of enzymic activity with IMP as the substrate (Table 1).

Reduction of the ribose moiety of GMP to deoxyribose also decreased the degree of inhibition; thus, dGMP was considerably less inhibitory than GMP. Nevertheless, dGMP was capable of producing relatively good inhibition of IMP dehydrogenase activity in the presence of a low concentration of IMP (2·3 $\times$ $10^{-5}$ M), but much less inhibition was observed at a 10-fold higher IMP concentration. Similarly, dIMP substituted for IMP as a substrate; however, the $V_{max}$ with this substrate was only 60 per cent as large as the $V_{max}$ for IMP. The apparent $K_m$ for dIMP was 4 $\times$ $10^{-4}$ M, a value about 20-fold higher than the apparent $K_m$ for IMP. Thus, replacement of the 2′-hydroxyl group of the nucleotide with hydrogen appeared to lower the effectiveness of both the substrate (IMP) and the feedback inhibitor (GMP).

Replacement of the carbon atom in position 8 of the purine ring of GMP by a nitrogen atom to form 8-azaGMP did not significantly alter the inhibition kinetics (Fig. 2). The data were linear and competitive in the double reciprocal plot and 8-azaGMP had a linear effect on the slope of the lines. The apparent $K_i$ for 8-azaGMP was 1·5 $\times$ $10^{-4}$ M, slightly lower than the apparent $K_i$ for GMP.

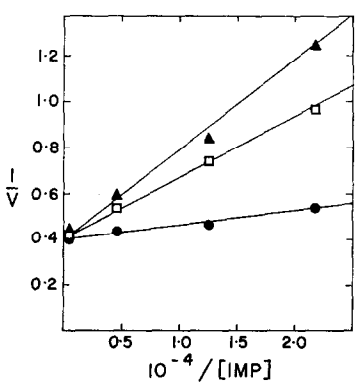


FIG. 2. Double reciprocal plot of the inhibition of the activity of IMP dehydrogenase by 8-azaguanosine 5′-phosphate (8-azaGMP). 8-AzaGMP was added to the preincubation mixture. The commercially obtained $Li^+$ salt of 8-azaGMP was converted to the $K^+$ salt by treatment with Dowex 50-$K^+$ prior to use to avoid possible inhibition by $Li^+$. V is initial velocity in mμmoles per min. Brackets indicate molar concentration. ●, No 8-azaGMP; □, 6·3 $\times$ $10^{-4}$ M 8-azaGMP; ▲, 1·3 $\times$ $10^{-3}$ M 8-azaGMP.

Alteration of the 6-position of the purine ring of GMP by substitution of an amino function appeared to have only slight effects on the inhibition kinetics; the findings depicted in Fig. 3 demonstrate that the ribonucleotide of 2, 6-diaminopurine (DAMP) produced linear plots of inhibition with primarily a slope effect. An additional small effect on the intercept, however, was detected. The apparent $K_i$ for DAMP, defined from the slope effect, was $1{\cdot}6 \times 10^{-4}$ M, a value similar to that for both GMP and 8-azaGMP. An intercept effect was more clearly demonstrated with the corresponding

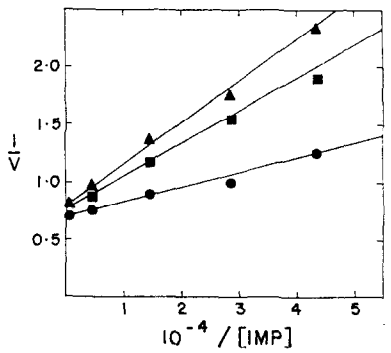


FIG. 3. Double reciprocal plot of the inhibition of IMP dehydrogenase by 2,6-diaminopurine ribonucleoside 5′-phosphate (DAMP). DAMP was added to the preincubation mixture. V is initial velocity in mμmoles per min. Brackets indicate molar concentrations. ●, No DAMP; ■, $1{\cdot}5 \times 10^{-4}$ M DAMP; ▲, $2{\cdot}9 \times 10^{-4}$ M DAMP.

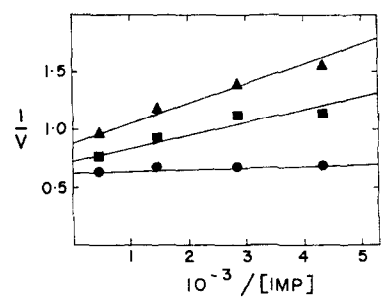


FIG. 4. Double reciprocal plot of the inhibition of IMP dehydrogenase by AMP. AMP was added to the preincubation mixture. V is initial velocity in mμmoles per min. Brackets indicate molar concentration. ●, No AMP; ■, $4{\cdot}9 \times 10^{-3}$ M AMP; ▲, $9{\cdot}8 \times 10^{-3}$ M AMP.

6-amino analog of the substrate; thus, AMP produced a distinctly noncompetitive pattern of inhibition (Fig. 4). Weaker inhibition of IMP dehydrogenase was induced by dAMP (Table 1), whereas ATP was inactive as an inhibitor at $10^{-3}$ M.

The pyrimidine nucleotides CMP, dCMP, CTP, dTMP, and UTP were inactive as inhibitors at $10^{-3}$ M; in contrast, UMP produced significant inhibition at low concentrations of IMP (Table 1).

From the results of investigations of the inhibition of IMP dehydrogenase of *A. aerogenes* by purine nucleotide analogs, Hampton *et al.*[2, 18, 19] presented ultraviolet spectral evidence that ClIMP inactivates the enzyme by reacting covalently with a

cysteine residue at the IMP binding site. Irreversible inactivation of the sarcoma 180 enzyme by purine nucleotide analogs[6,20] suggested the presence of a similar reactive group at the IMP binding site of the sarcoma 180 enzyme and led to an attempt to use the reactivity of this group to determine whether GMP interacts with the IMP binding site of IMP dehydrogenase of sarcoma 180. IMP dehydrogenase was inhibited by *N*-ethylmaleimide (Table 2), as well as by iodoacetic acid, iodoacetamide, and *p*-hydroxymercuribenzoate; the results with *N*-ethylmaleimide support the contention that the

TABLE 2. PROTECTION OF IMP DEHYDROGENASE BY SEVERAL NUCLEOTIDES FROM INHIBITION BY *N*-ETHYLMALEIMIDE*

| Protecting agent | Concn during preincubation (mM) | Percentage inhibition |
|---|---|---|
| NONE | | 81 |
| IMP | 0·13 | 72 |
| | 0·26 | 55 |
| | 1·3 | 38 |
| | 2·6 | 17 |
| XMP | 0·13 | 71 |
| | 0·26 | 47 |
| | 1·3 | 23 |
| | 5·2 | 13 |
| GMP | 0·26 | 75 |
| | 1·3 | 58 |
| | 2·6 | 48 |
| | 7·8 | 48 |

* IMP dehydrogenase was preincubated for 5 min at 37° with $2{\cdot}9 \times 10^{-5}$ M *N*-ethylmaleimide in 0·9 ml of 0·1 M Tris-Cl, pH 8, 0·1 M KCl, and the indicated concentrations of IMP, XMP, and GMP. At the end of the preincubation time, $2{\cdot}3 \times 10^{-3}$ M IMP and $2{\cdot}8 \times 10^{-4}$ M $NAD^+$, prewarmed to 37°, were added in a volume of 0·1 ml, and the residual enzymatic activity was measured during the next 10 min. The percentage inhibition was determined by comparing the activity in the presence of *N*-ethylmaleimide and nucleotides with activities which were obtained after preincubation of the enzyme with the nucleotides in the absence of *N*-ethylmaleimide.

reactive group on the mammalian enzyme might well be a sulfhydryl function. The presence of either IMP or the enzymatic reaction product XMP during preincubation of the enzyme with *N*-ethylmaleimide reduced the rate of inactivation of enzymatic activity; the protection was proportional to the concentration of purine nucleotide. Similar results were obtained with iodoacetic acid and iodoacetamide. GMP was also able to protect IMP dehydrogenase activity from the inhibitory effects of *N*-ethylmaleimide (Table 2).

The nucleotide analog 6-chloropurine ribonucleoside 5′-phosphate (ClIMP) progressively inhibited the activity of IMP dehydrogenase of sarcoma 180; furthermore, both IMP and XMP completely protected the enzyme from inactivation by ClIMP.[6,21] Similarly, GMP completely protected the enzyme from inactivation by ClIMP; a

concentration of GMP approximating its $K_i$ value produced about 50 per cent protection (Fig. 5).

The activity of IMP dehydrogenase was also inhibited by 2-amino-6-chloropurine ribonucleoside 5′-phosphate (ClGMP).[22] This inhibition was enhanced by preincubation of the inhibitor with the enzyme in the absence of IMP and $NAD^+$. Thus, the presence of IMP and $NAD^+$ prevented the attainment of maximal inhibition by ClGMP (Table 3). Inhibition was specific for the ribonucleotide of 2-amino-6-chloropurine, since $3{\cdot}8 \times 10^{-3}$ M 2-amino-6-chloropurine and $4{\cdot}5 \times 10^{-3}$ M 2-amino-6-chloropurine ribonucleoside produced 0 and 21 per cent inhibition, respectively, of enzymatic activity after preincubation of these agents with the enzyme in the absence of substrates. Dialysis of the enzyme–ClGMP mixture resulted in only minimal reversal of inhibition. The findings with ClGMP are similar to the effects produced by

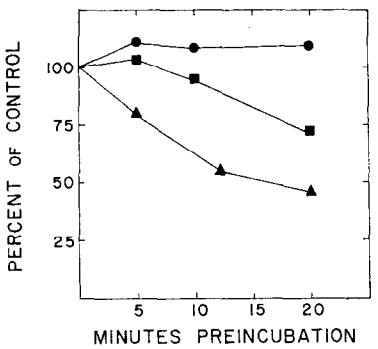


FIG. 5. Inhibition of IMP dehydrogenase activity by 6-chloropurine ribonucleoside 5′-phosphate (ClIMP). ClIMP ($7{\cdot}6 \times 10^{-5}$ M) was preincubated with IMP dehydrogenase in a total volume of 0·9 ml of 0·1 M Tris-Cl, pH 8, and 0·11 M KCl at 37° with the following additions: ●, $2{\cdot}6 \times 10^{-3}$ M GMP; ■, $2{\cdot}6 \times 10^{-4}$ M GMP; ▲, no GMP. At the indicated times of preincubation, 0·1 ml of a substrate mixture, prewarmed to 37°, was added to bring the concentration of IMP to $2{\cdot}3 \times 10^{-3}$ M and of $NAD^+$ to $2{\cdot}8 \times 10^{-4}$ M. The residual enzymatic activity was measured during the next 10 min and expressed relative to the activity obtained in the presence of GMP and absence of ClIMP.

TABLE 3. INHIBITION OF IMP DEHYDROGENASE BY 2-AMINO-6-CHLOROPURINE RIBONUCLEOSIDE 5′-PHOSPHATE (ClGMP)*

| 2-Amino-6-chloropurine ribonucleoside 5′-phosphate (mM) | Percentage inhibition | |
|---|---|---|
| | No preincubation | Preincubation |
| 0·25 | 34 | 57 |
| 0·44 | 59 | 76 |
| 0·62 | 72 | 83 |

* A preparation of IMP dehydrogenase (2·16 mg of protein) was preincubated at 37° in a total volume of 0·9 ml containing 0·1 M Tris-Cl, pH 8, 0·11 M KCl, and ClGMP (the concentrations of ClGMP listed in the Table refer to those added at the beginning of the preincubation). After 10 min, 0·1 ml of a substrate mixture was added to bring the concentration of IMP to $2{\cdot}3 \times 10^{-3}$ M and $NAD^+$ to $2{\cdot}8 \times 10^{-4}$ M, and the residual enzymatic activity was measured during the next 10 min. The data for no preincubation were obtained by addition of IMP and $NAD^+$ 3 min prior to the addition of the ClGMP.

ClIMP on the activity of this enzyme;[6] thus, the GMP analog, ClGMP, like the IMP analog, ClIMP, reacts with the enzyme in a relatively stable manner at a site(s) at which the substrates afford at least partial protection of the enzyme from the inhibitor.

Several attempts were made to desensitize IMP dehydrogenase to the inhibition produced by GMP. Preincubation of the enzyme at 55° in 0·01 M Tris-Cl (pH 8) for up to 48 min, addition of 5% ethanol, or addition of 0·4 M urea failed to significantly affect the degree of inhibition produced by GMP. However, addition of 0·1 M or 0·4 M phosphate ($K^+$ salt; pH 8) caused less than a 10 per cent lowering of the enzymatic activity, while reducing the inhibition produced by GMP by 30 to 40 per cent. Greater desensitization was not achieved.

## DISCUSSION

Studies on the mechanism by which the purine nucleotide GMP functions to control its own synthesis by inhibition of the activity of IMP dehydrogenase has been investigated in several microbial systems. In *A. aerogenes*, GMP is kinetically competitive with IMP,[2] while in *S. typhimurium* and *B. subtilis* double reciprocal plots of IMP concentration versus initial velocity demonstrate nonlinearities with GMP as an inhibitor.[3,4] In the present studies, linear double reciprocal plots were obtained with an IMP dehydrogenase preparation of mammalian origin (sarcoma 180) in both the absence and presence of either GMP, 8-azaGMP, DAMP, or AMP. Buzzee and Levin[3] have emphasized the necessity of careful preparation of the enzyme to demonstrate the nonlinear effects obtained in the presence of GMP which are ascribed to allosteric phenomena. The preparation of the sarcoma 180 enzyme was relatively gentle and similar to the procedure employed by Buzzee and Levin;[3] therefore, it would not appear that instability of an effector site was responsible for the differences in the kinetic behavior observed with these two enzymatic preparations. The variability between experiments in the $K_i$ for GMP (i.e. 2 to 8 $\times$ $10^{-4}$ M) with the mammalian enzyme and the partial desensitization obtained with potassium phosphate are the only indications that the GMP site might be subject to alteration independent of the IMP site.

Similarities were observed in the structural specificity of the GMP and IMP binding site(s). Thus, the effectiveness of IMP as a substrate and of GMP as an inhibitor was reduced by alteration of the 2′-position (i.e. substitution of dIMP and dGMP respectively) and the 5′-position (i.e. substitution of inosine diphosphate and guanosine diphosphate respectively). It is not known whether the apparent stimulation of enzymatic activity by GDP (Table 1) is the result of a specific effect of this compound or is attributable to a contaminant. The inhibition kinetics obtained with DAMP, which may be considered to be a derivative of GMP aminated in the 6-position, differed only slightly from those obtained with GMP, while the inhibition produced by AMP, the corresponding aminated derivative of IMP, consisted of a much larger element of noncompetition. Although the kinetics of inhibition by AMP have not been determined with $NAD^+$ as the variable substrate, it is likely that the noncompetitive aspect of the AMP inhibition arises from the structural similarity between AMP and the adenine nucleotide portion of the $NAD^+$ molecule, and occurs at the $NAD^+$ binding site. Indeed, a similar concentration of $NAD^+$ produces a corresponding degree of inhibition.[7] Alternately, a separate binding site for AMP might exist; however, the biochemical utility of such a negative effector site is not apparent. The kinetic differences

between AMP and DAMP do not necessarily imply a difference between hypothetical IMP and GMP binding sites with respect to structural specificity.

IMP, XMP,[6,21] and GMP (Table 2) behaved qualitatively in a similar manner in protecting the enzymatic activity from inhibition by *N*-ethylmaleimide and ClIMP, both of which might be envisioned to react with a sulfhydryl group at the active site of the enzyme. However, the concentrations of IMP, XMP and GMP required to significantly delay inactivation by *N*-ethylmaleimide are high relative to their respective apparent $K_m$ and $K_i$ values [$K_m$ (IMP) $= 2 \times 10^{-5}$ M; $K_i$ (XMP) $= 4 \times 10^{-4}$ M; $K_i$ (GMP) $= 2$ to $8 \times 10^{-4}$ M]. Investigations of initial velocity and product inhibition patterns for IMP dehydrogenase have indicated that IMP is probably the first substrate to bind to the enzyme in the biosynthetic reaction;[6,7,20] thus, the apparent $K_m$ for IMP should represent a true dissociation constant. Therefore, the requirement for relatively high concentrations of IMP to protect the enzyme from the inhibitory actions of *N*-ethylmaleimide casts doubt as to whether such effects occur at the catalytic site of the enzyme. The concentrations of IMP, XMP,[6,21] and GMP (Fig. 5) required to delay inactivation of enzymic activity by ClIMP, on the other hand, are more closely related to the apparent $K_m$ of IMP and $K_i$ values of XMP and GMP. If a two-stage reaction of the enzyme with ClIMP is assumed,[21] the first reversible and the second irreversible, then the actual effectiveness of the purine nucleotides as protecting agents should depend not only on their concentrations relative to their $K_m$ and $K_i$ values, but also on the concentration of ClIMP relative to its apparent reversible binding constant. Good agreement exists between the predicted and observed protection of the inactivation of IMP dehydrogenase by ClIMP[21] by IMP, XMP and GMP. The protection of IMP dehydrogenase afforded by IMP and GMP against inactivation by ClIMP is the most significant finding which suggests that the IMP and GMP binding sites are identical. These data, however, do not eliminate the possibility that GMP reacts with an allosteric site to alter the affinity of the active site for both IMP and its analog, ClIMP.

The GMP analog, ClGMP, produced progressive irreversible changes in the enzyme in the absence of substrates in a manner similar to that caused by the IMP analog, ClIMP. Two differences, however, were noted between ClIMP and ClGMP in this system. First, the latter compound inhibited enzymatic activity in the presence of substrates, whereas ClIMP required preincubation with the enzyme in the absence of substrates to be inhibitory. Second, $K^+$, the activator of IMP dehydrogenase, accelerated inactivation of the enzyme by ClIMP, but had no effect on the rate of inactivation by ClGMP;* the significance of these differences is unknown.

Thus, although certain differences exist between IMP, ClIMP, GMP and ClGMP in their patterns of reaction with the IMP dehydrogenase of sarcoma 180 cells, no evidence was found which is incompatible with the involvement of only a single binding-site on this enzyme for all of these compounds.

* J. H. Anderson and A. C. Sartorelli, unpublished observations.

## REFERENCES

1. J. MAGER and B. MAGASANIK, *J. biol. Chem.* **235**, 1474 (1960).
2. A. HAMPTON and A. NOMURA, *Biochemistry* **6**, 679 (1967).
3. D. H. BUZZEE and A. P. LEVIN, *Biochem. biophys. Res. Commun.* **30**, 673 (1968).
4. K. ISHII and I. SHIIO, *J. Biochem.* **63**, 661 (1968).

5. J. H. ANDERSON and A. C. SARTORELLI, *Fedn Proc.* **25**, 745 (1966).
6. J. H. ANDERSON and A. C. SARTORELLI, *Fedn Proc.* **26**, 730 (1967).
7. J. H. ANDERSON and A. C. SARTORELLI, *J. biol. Chem.* **243**, 4762 (1968).
8. B. MAGASANIK, H. S. MOYED and L. B. GEHRING, *J. biol. Chem.* **226**, 339 (1957).
9. W. W. CLELAND, *Nature, Lond.* **198**, 463 (1963).
10. E. RANDERATH and K. RANDERATH, *Analyt. Biochem.* **12**, 83 (1965).
11. A. HAMPTON and D. I. MAGRATH, *J. Am. chem. Soc.* **79**, 3250 (1957).
12. J. A. JOHNSON and H. J. THOMAS, *J. Am. chem. Soc.* **78**, 3863 (1956).
13. N. ANAND, V. M. CLARKE, R. H. HALL and A. R. TODD, *J. chem. Soc.* p. 3365 (1950).
14. J. A. MONTGOMERY and H. J. THOMAS, *J. org. Chem.* **26**, 1926 (1961).
15. J. K. ROY, D. C. KVAM, J. L. DAHL and R. E. PARKS, JR., *J. biol. Chem.* **236**, 1158 (1961).
16. J. L. WAY and R. E. PARKS, JR., *J. biol. Chem.* **231**, 467 (1958).
17. A. HAMPTON and M. H. MAGUIRE, *J. Am. chem. Soc.* **83**, 150 (1961).
18. A. HAMPTON, *J. biol. Chem.* **238**, 3068 (1963).
19. L. W. BROX and A. HAMPTON, *Biochemistry* **7**, 2589 (1968).
20. R. P. MIECH, R. E. PARKS, JR., J. H. ANDERSON and A. C. SARTORELLI, *Biochem. Pharmac.* **16**, 2222 (1967).
21. J. H. ANDERSON and A. C. SARTORELLI, *Biochem Pharmac.* **18**, 2737 (1969).
22. A. C. SARTORELLI, J. H. ANDERSON and B. A. BOOTH, *Biochem. Pharmac.* **17**, 37 (1968).