Inosine 5′-Phosphate Dehydrogenase. Site of Inhibition by Guanosine 5′-Phosphate and of Inactivation by 6-Chloro- and 6-Mercaptopurine Ribonucleoside 5′-Phosphates*

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ABSTRACT: Inosine 5′-phosphate dehydrogenase of *Aerobacter aerogenes* has been purified 150-fold. The procedures freed the preparation of guanosine 5′-phosphate reductase, reduced nicotinamide–adenine dinucleotide (NADH) oxidase activity, and activities (possibly of the adenosine deaminase type) which convert 6-chloropurine ribonucleoside and its 5′-phosphate to inosine and inosine 5′-phosphate, respectively. At pH 8.0 and 25° the Michaelis constant of both inosine 5′-phosphate and 6-thioinosine 5′-phosphate was 2.1×10^{-5} M and that of nicotinamide–adenine dinucleotide (NAD) was 1.1×10^{-3} M. The value for inosine 5′-phosphate was independent of NAD concentration and the value for NAD was independent of inosine 5′-phosphate concentration and hence both values presumably represent enzyme–substrate dissociation constants. The Michaelis constants of inosine 5′-phosphate and 6-thioinosine 5′-phosphate were essentially constant in the pH range 6.5–8.2 and decreased sharply at higher pH values. The Michaelis constant of NAD decreased in two or more increments over the same pH range. The maximum initial velocity for conversion of inosine 5′-phosphate to xanthosine 5′-phosphate was constant from pH 8 to 9 and progressively decreased from pH 8 to 6.5. Guanosine 5′-phosphate was a competitive inhibitor with respect to inosine 5′-phosphate; the inhibitor constant was 1.3×10^{-4} M. Inosine 5′-phosphate and guanosine 5′-phosphate retarded inactivation of the enzyme by 6-chloropurine ribonucleoside 5′-phosphate; their relative effectiveness (6.5:1) was the same as the ratio of their respective inhibitor and Michaelis constants, and hence guanosine 5′-phosphate evidently inhibits the conversion of inosine 5′-phosphate to xanthosine 5′-phosphate by reversible attachment to the inosine 5′-phosphate site. In the absence of glutathione, low concentrations of 6-thioinosine 5′-phosphate rapidly inactivate the enzyme. The reaction is retarded by inosine 5′-phosphate but not by NAD. The effectiveness of 6-thioinosine 5′-phosphate relative to that of inosine 5′-phosphate for retarding inactivation by the 6-chloropurine nucleotide underwent a 1000-fold increase when glutathione was omitted.

The present results lend further support to the conclusion that the 6-chloro- and 6-thionucleotides inactivate the enzyme by reacting at the inosine 5′-phosphate site.

Low concentrations of the 6-chloro and 6-mercapto analogs of inosine 5′-phosphate rapidly inactivate IMP dehydrogenase[1] of *Aerobacter aerogenes*, and evidence indicates that the two analogs exert this effect at the IMP reaction site, apparently by forming thioether and disulfide bonds, respectively, with an enzymic sulfhydryl group (Hampton, 1963). This report describes a method by which the IMP dehydrogenase can be freed of NADH-oxidase activity, dechlorinase[2] activity toward 6-chloropurine nucleoside[3] and nucleotide, and GMP reductase (EC 1.6.6.8); this last enzyme readily reacts covalently with 6-chloropurine nucleotide and 6-thio-IMP (Hampton *et al.*, 1966). The IMP dehydrogenase so purified has been employed for studies of substrate and inhibitor kinetics which have yielded further evidence that the 6-chloro and 6-mercapto analogs of IMP react covalently at the IMP site of the enzyme.

It appears likely that inhibition of IMP dehydrogenase by GMP is involved in the regulation of GMP biosynthesis in bacteria (Mager and Magasanik, 1960; Magasanik and Karibian, 1960). The present studies indicate that this inhibition is of the classical competitive type and occurs at the IMP site itself rather than at an alternative (regulatory) site.

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[1] IMP:NAD oxidoreductase, EC 1.2.1.14.

[2] This denotes replacement of a chlorine by hydroxyl.

[3] Abbreviations: 6-thio-IMP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-phosphate; 6-chloropurine nucleotide, 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate; IMP, XMP, and GMP, inosine, xanthosine, and guanosine 5′-phosphates; NAD, nicotinamide–adenine dinucleotide; NADH, reduced NAD; TPNH, reduced triphosphopyridine nucleotide.

Materials and Methods

Chemicals were obtained from the sources listed previously (Hampton, 1963).

Enzyme Purification. A. aerogenes, strain P-14, grown in liquid medium containing 10 mg/l. of guanine (Magasanik *et al.*, 1957) was obtained from the Miles Chemical Co., Elkhart, Ind. The use of less guanine in the medium reduced the yield and specific activity of the enzyme (purified to step 3 below). The frozen cells were thawed in an equal volume of 0.03 M potassium phosphate buffer, pH 7.4, and collected by centrifugation. The cells were rewashed and suspended in 10 volumes of the buffer. Portions (250 ml) of the cell suspension in 500-ml beakers were cooled in an ice bath and sonicated with the maximum output of a Branson Sonifier, Model S-75 (20 kcycles, 75 w), four times in 5-min periods. The top of the sonicator probe was positioned 1.5 cm from the bottom of the beaker. The combined sonicates were stored in the cold room at 2° overnight and centrifuged.

All procedures were carried out at 0–4°. Except for the removal of the precipitates from protamine treatment, in which an International clinical centrifuge was used, centrifugations were performed in a Servall R-2 centrifuge for 15 min at 23,500*g*. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (fraction V, Sigma Chemical Co.) as standard.

Chromatography of Enzymes on Phosphocellulose. Whatman phosphocellulose (7.4 mequiv/g) was prepared according to the general procedure of Peterson and Sober (1962) and packed under 5 psi into a Kontes Chromatoflex column (inner diameter 2.5 cm) which contained a Teflon supporting disk. The packed column, about 20 cm in height, was equilibrated with 0.02 M potassium phosphate buffer, pH 7.4.

Ammonium sulfate fraction (10–15 ml) (step 3) was dialyzed against 1 l. of the buffer for about 16 hr and applied immediately to the column. The column was washed with buffer until weakly adsorbed protein had passed through (about 150 ml). Fractions of 10 ml were collected at a flow rate of 40 ml/hr. Fractions containing high enzyme activity were combined and stored in polyethylene containers at −20°. The chromatographic properties of other ion-exchange celluloses were tested by application of 5–7 mg of protein from the ammonium sulfate fraction to 1 × 20 cm columns.

Assay of GMP Reductase. The assay system contained 40 μmoles of Tris-HCl buffer, pH 7.5, 2 μmoles of glutathione, 0.2 μmole of GMP, 0.2 μmole of TPNH, and enzyme in a total volume of 1.0 ml. Reaction was started by addition of GMP and followed by the decrease in optical density at 350 mμ. One unit of enzyme was defined as that amount causing an optical density change of 1.00 in 1 min.

Assays of IMP Dehydrogenase. (a) In purification experiments, enzyme activities were measured by following the increase in optical density at 290 mμ due to the conversion of IMP to XMP. Each cuvet (1.0-cm light path) contained 25 μmoles of Tris-HCl buffer, pH 8.0, 25 μmoles of KCl, 2 μmoles of glutathione, 0.5 μmole of NAD, and enzyme. The mixture was placed in a Cary Model 15 spectrophotometer and after 5 min the reaction was started by addition of 0.125 μmole of IMP, which brought the final volume to 1.0 ml. The resulting change in optical density was recorded at 23–25° against a blank which lacked IMP. The arbitrary unit of enzyme activity was defined as that which effected an increase in optical density of 1.00 in 1 min.

(b) Kinetic experiments were carried out with the enzyme preparation purified by phosphocellulose chromatography (step 4, Table I). The enzyme solutions employed had been once frozen and were freshly thawed. Each 1-cm light path cuvet contained 25 μmoles of Tris-citrate buffer, pH 8.5, 100 μmoles of KCl, 2 μmoles of glutathione, 1 μmole of NAD, and enzyme. Cuvets were placed in a thermostated cell compartment (25°) of a Cary Model 15 spectrophotometer and after 5 min the reaction was started by addition of 0.25 μmole of IMP. The final volume was 1.0 ml. The increases in optical density at 290 mμ (owing to the conversion of IMP to XMP) or at 340 or 350 mμ (owing to the formation of NADH)

TABLE I: Purification of IMP Dehydrogenase.

Step	Fraction	Vol. (ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg of protein)	Purification
1	Crude extract[a]	497	2982	517	0.170	1.00
2	Protamine sulfate	508	2286	879	0.384	2.26
3	Ammonium sulfate	35	788	788	1.000	5.89
4	Phosphocellulose[b]	93	82	392	4.78	28.1
5a	DEAE-cellulose		10.3[c]	275	26.8	157
5b	2nd ammonium sulfate		19.4	333	17.2	101

[a] From 60 g of washed packed cells. [b] The data were converted from those obtained in an experiment with 15 ml of ammonium sulfate fraction. [c] Protein was determined from absorption at 280 mμ.

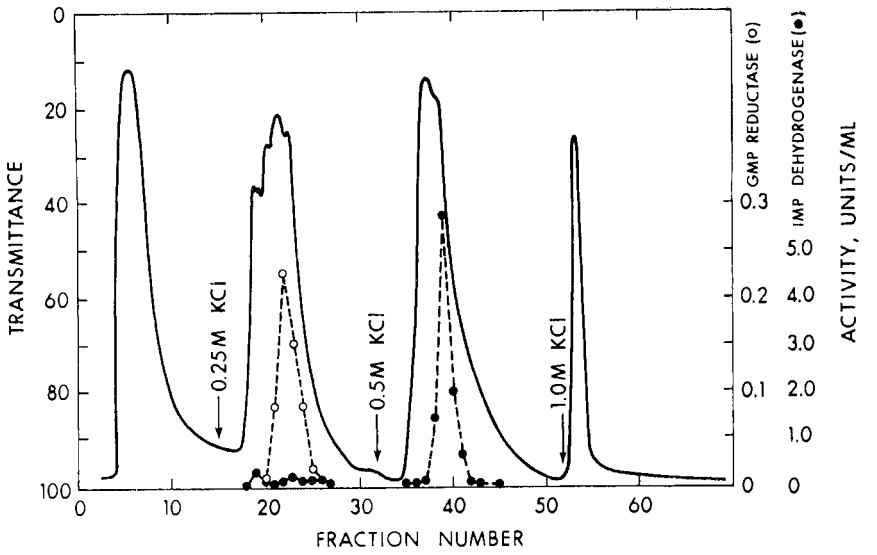

FIGURE 1: Elution of IMP dehydrogenase and GMP reductase from phosphocellulose. The column (2.5 × 18 cm) was loaded with 340 mg of step 3 protein. The solid line represents transmittance at 280 mμ. The arrows indicate stepwise changes in KCl concentration.

were recorded for at least 10 min against a blank which lacked IMP. Initial velocity was expressed as the change in optical density in 10 min. Enzyme inhibitors and protectors were added to the assay system before addition of the enzyme. When IMP was employed as a protector, the final addition was NAD. In some experiments the reaction was started by the simultaneous addition of glutathione with either IMP or NAD. In all such experiments, initial concentrations of components were never more than 10% higher than their final concentrations. When 6-thio-IMP was the substrate the absorbancy scale of the spectrophotometer was expanded tenfold and four times more enzyme was employed; the final component added was NAD. The pH of reaction mixtures was determined with a Corning Model 12 pH meter; the above assay system had pH 8.01 at 25°.

Kinetic data were analyzed graphically by the method of Lineweaver and Burk (1934). K_i for a competitive inhibitor was calculated from the equation $K_p = K_m(1 + [\text{inhibitor}]/K_i)$, where K_p is the apparent value of K_m obtained in the presence of inhibitor.

Results

Purification of IMP Dehydrogenase. The enzyme activity obtained from bacterial suspensions reached a constant value after sonication for 15 min under the conditions described. The enzymic activity of the sonicates increased two- to threefold after overnight storage at 2°. Longer storage did not further enhance activity. Inclusion of β-mercaptoethanol or glutathione (1 mM) in the cell suspensions did not affect this process. The protamine sulfate and ammonium sulfate treatments (Table I) were carried out as described by Magasanik *et al.* (1957), except that solid ammonium sulfate was used instead of a saturated solution. In some experiments, 30% saturation with ammonium sulfate caused no precipitation and a centrifugation at this stage was accordingly omitted.

When eluted from phosphocellulose by stepwise increases in KCl concentration, IMP dehydrogenase was purified about fivefold and freed of GMP reductase, NADH oxidase(s), and a fraction with dechlorinase activity toward 6-chloropurine nucleotide (Figure 1). The recovery of IMP dehydrogenase activity from the column was about 50% of that present in the step 3 fraction. During the dialysis which preceded chromatography the preparation lost 30% of its activity. Application of a linear gradient of KCl to the phosphocellulose column resulted in a broadening and overlapping of the peaks containing IMP dehydrogenase and GMP reductase.

Dialyzed step 3 protein was applied to carboxymethylcellulose in phosphate buffer at pH 6.4 and 7.4, but in both cases the IMP dehydrogenase, which was not retained, was not appreciably purified. Significant purification of the step 3 fraction could be achieved by chromatography on DEAE cellulose in the pH range 6.4–8.0, when all the protein was adsorbed and 70% of the activity was eluted with 0.5 M KCl. Traces of ammonium sulfate in the step 3 fraction prevented adsorption of protein on the DEAE-cellulose, and prior removal of ammonium sulfate with a Sephadex G-25 column was necessary for enzyme purification.

In step 5a (Table I), IMP dehydrogenase (13 units) from the phosphocellulose column was dialyzed overnight against 0.05 M potassium phosphate, pH 6.4, and put on a 1 × 19 cm column of DEAE-cellulose which had been equilibrated with a mixture of the same buffer and 1 mM β-mercaptoethanol. Elution

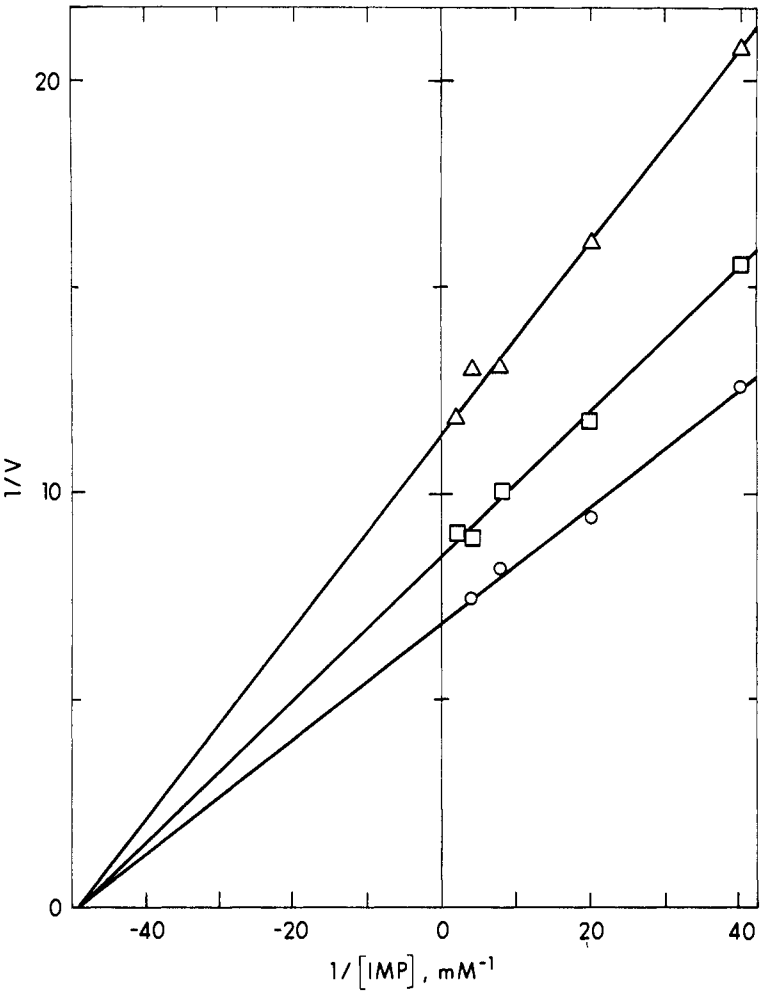

FIGURE 2: Lineweaver–Burk plots of initial velocity *vs.* IMP concentration at the following three constant levels of NAD: 0.5 mM (△), 1.0 mM (□), and 2.0 mM (○). Velocities were measured at 290 mμ. Reaction mixtures (see text) contained 2.2 μg of step 4 protein.

was performed with a linear gradient of KCl (0–1 M with 200 ml of eluent) in the phosphate buffer–mercaptoethanol mixture. IMP dehydrogenase appeared as a single relatively symmetrical peak at a KCl concentration of about 0.2 M. The recovery of enzyme activity was 70–80%.

In an alternative purification procedure (step 5b) the phosphocellulose fraction was dialyzed overnight against 0.02 M potassium phosphate (pH 6.4) and ammonium sulfate was added. Between 30 and 45% saturation, 85% of the enzyme activity precipitated and a 3.6-fold increase in specific activity resulted.

A cellulose derivative was prepared by coupling 6-thioguanosine at its 8 position with diazotized *p*-aminobenzylcellulose (Bio-Rad Laboratories) and was tested as a means for further purification. It was hoped that the IMP dehydrogenase could become selectively adsorbed by formation of a disulfide bond with the thioguanosine (Hampton, 1963) and subsequently be released by elution with an aqueous solution of a mercaptan. However, this cellulose derivative adsorbed all proteins in the step 4 preparation, and attempts to elute the enzyme were not successful. Conditions tested included increase and decrease in KCl or $(NH_4)_2SO_4$ concentration (0–1 M) and elution with 1–10 mM β-mercaptoethanol. When relatively small amounts of the thioguanosine-cellulose were employed, the specific activity of unabsorbed enzyme was not altered.

Stability of the Enzyme. The step 4 enzyme fraction lost about 20% of its activity after 2 weeks at 2°; thereafter activity was lost more rapidly. Step 4 enzyme lost activity more rapidly when diluted with water, Tris, or phosphate buffer. After a 1:20 dilution with water, for example, 50% of the activity was lost in 2–3 days at 2°. The diluted enzyme was more unstable at pH 8 and 9 than at pH 7. KCl (0.1–0.5 M), $(NH_4)_2SO_4$ (0.1 M), glutathione (2 mM), and β-mercaptoethanol (1 mM) had no stabilizing effect. Higher concentrations of glutathione (10 and 50 mM) were inhibitory (23 and 86%, respectively). Dilution 1:20 with bovine serum albumin (final concentration 0.05%) stimulated enzyme activity about 50%.

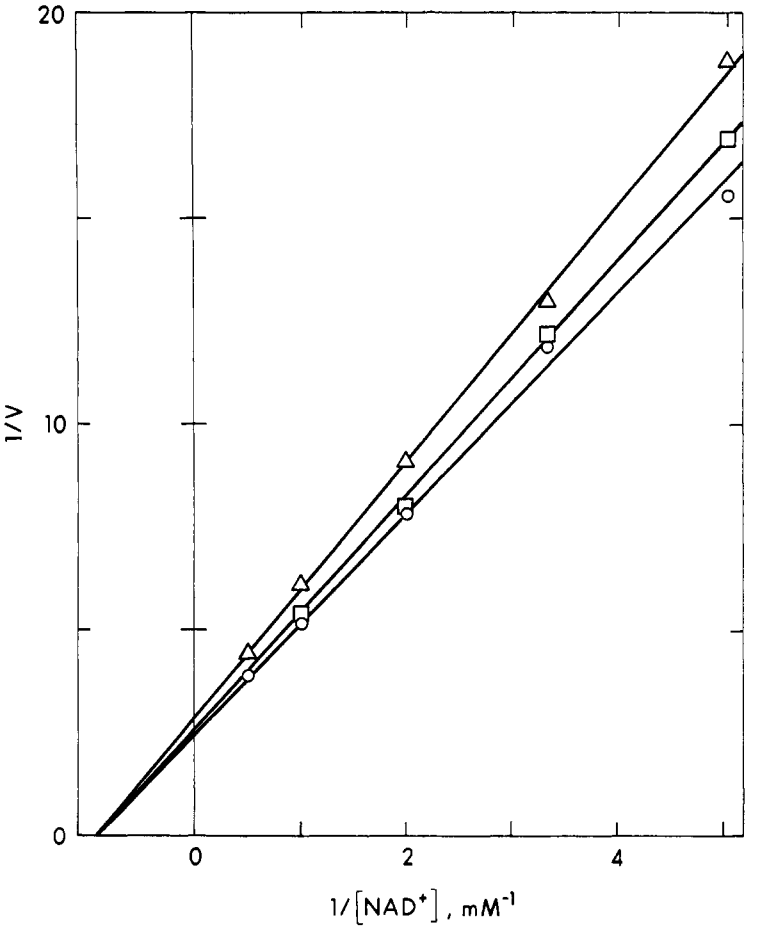

FIGURE 3: Lineweaver–Burk plots with NAD for three constant concentrations of IMP, namely, 0.1 mM (△), 0.25 mM (□), and 0.5 mM (○). Other conditions were as in Figure 2, except that 2.9 μg of protein was employed.

The step 4 enzyme lost about 50% of its activity when initially frozen, but further inactivation did not occur after several months at −20° nor after repeated thawing and freezing. The loss of activity upon initial freezing was not prevented by the presence of 1 mM β-mercaptoethanol.

The step 5a enzyme preparation (in 0.2 M KCl) was not inactivated by freezing and maintained its activity when stored for 3 weeks at −20°, during which time it was thawed and frozen twice and kept at 2° for two 24-hr periods.

Assay for NADH Oxidase. In some experiments with IMP dehydrogenase, for example those with high concentrations of NAD or 6-thio-IMP, a high blank absorption interfered with measurement of the optical density change at 290 mμ. To test the feasibility of measuring the rate of reaction at 340 or 350 mμ, the level of NADH oxidase(s) in the phosphocellulose fraction was measured. With fivefold more of enzyme than that employed in most of the kinetic experiments, no decrease of absorption at 340 mμ occurred over a period of 1 hr after the conversion of NAD to NADH was complete. This absence of detectable NADH oxidase activity was observed at pH values of 7.5 and 9.0. At pH 6.5, and with 50 times more enzyme than usual, a slow decrease of absorption at 340 mμ was observed after the maximum had been reached. The rate of this decrease was 1.1% that of the initial increase.

Effect of pH. The optimum pH of the enzyme (step 4 preparation) was determined under three conditions: (a) the assay system used for the purification experiments, (b) the assay system used for kinetic experiments, and (c) system b containing 0.05% bovine serum albumin. Under all conditions the activity *vs.* pH curves were essentially the same and showed pH optima of 8.2. A value of 8.1 was reported by Magasanik *et al.* (1957).

Cofactor Requirements. IMP dehydrogenase of *A. aerogenes* has been shown to require K^+ or NH_4^+ and a sulfhydryl compound (Magasanik *et al.*, 1957). Cyanide ion can substitute for a sulfhydryl compound with enzyme from this source and has been concluded to act by cleaving enzymic disulfide bonds (Weinbaum and Suhadolnik, 1964). The step 4 preparations of the present work (in the presence or absence of 0.05% bovine serum albumin) required 0.1 M KCl for maximum activity, and higher concentrations were inhibitory.

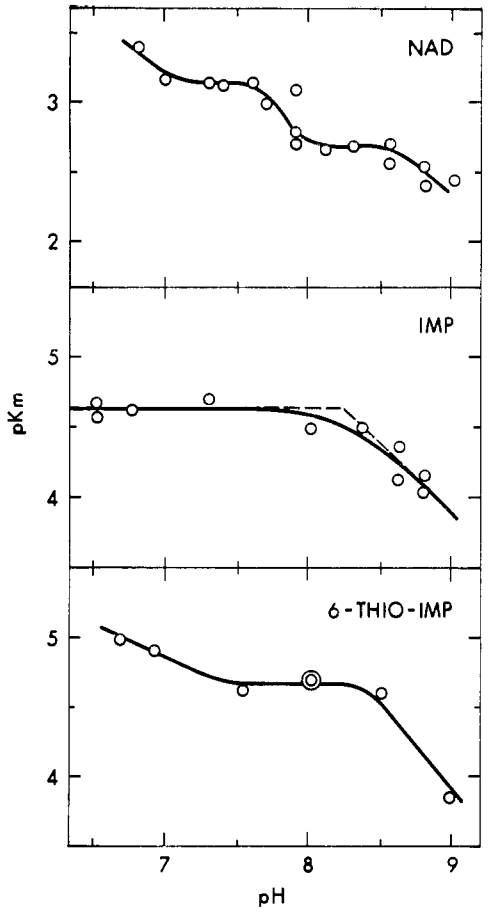

FIGURE 4: Effect of pH on the pK_m of NAD, IMP, and 6-thio-IMP. The amount of step 4 protein used at the different pH values varied from 2.2 to 8.8 μg/assay according to the respective reaction rates. Other conditions are given under Methods. Initial velocity was measured at 350 mμ.

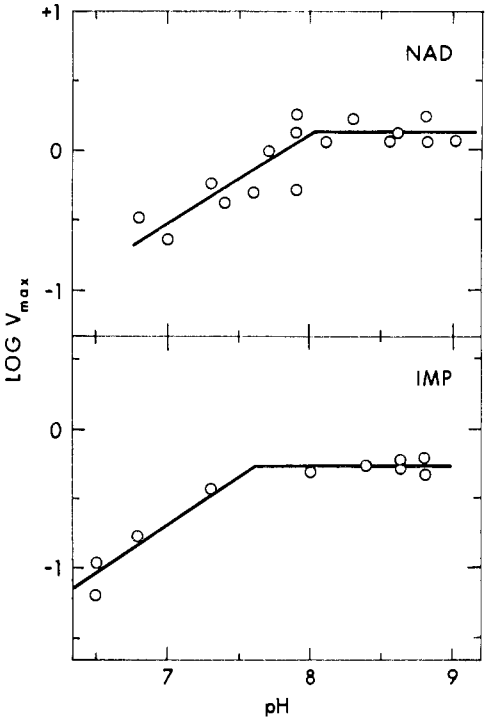

FIGURE 5: Effect of pH on the V_{max} values of IMP dehydrogenase. The data were obtained from the experiments of Figure 4. V_{max} values have been corrected where necessary so as to refer to reaction mixtures which contain 4.4 μg of protein.

The level of glutathione for maximum activity was between 2 and 4 mM. Higher concentrations were inhibitory (see above, under Stability). In the absence of glutathione the activity of various step 4 preparations was 30–50% of maximal. IMP dehydrogenase from Sarcoma 180 cells and Ehrlich ascites cells is not stimulated by glutathione (Anderson and Sartorelli, 1966; Atkinson *et al.*, 1963).

Substrate Kinetics. The Michaelis constant (K_m) for IMP at pH 8.0 and 25° was obtained from initial velocity analyses with IMP as a variable substrate and NAD as a changing fixed substrate. With three different concentrations of NAD, a single K_m value of 2.1×10^{-5} M for IMP was obtained (Figure 2). The K_m of IMP at pH 8.0, 25°, under the conditions of assay b but in the absence of glutathione was 2.12×10^{-5} and 2.15×10^{-5} M in two separate determinations. Magasanik *et al.* (1957) also reported that the K_m of IMP was independent of glutathione concentration. When NAD was the variable substrate and three levels of IMP were employed, a single K_m value of 1.1×10^{-3} M for NAD was obtained (Figure 3).

Figure 4 shows the effect of pH on Michaelis constant. In line with current kinetic analysis and data from other systems (Dixon and Webb, 1964), the plots of Figure 4 consist in the main of sequences of straight lines with slopes of zero or a whole number (in this case, −1). In the case of IMP, the K_m value was constant over the pH range of 6.5–8.0 and underwent a sharp decrease at higher pH values. Such a negative inflection corresponds to ionization of a group which is involved in formation of the enzyme–substrate complex and which can be located in either the free enzyme or the free substrate (Dixon and Webb, 1964). The pK of this group is given by the point of intersection of the two straight lines and has a value of about 8.3.

The pK_m plot for NAD (Figure 4) was more complex and showed pK values of 7.7 and 8.6, respectively, for two groups, either of which could be in the enzyme or the substrate; in addition, an upward bend of the graph at pH 8 corresponds to a group of pK = 8 in the enzyme–substrate complex.

The effect of pH on V_{max} reflects the dissociation of the enzyme–substrate complex (Dixon and Webb, 1964). In Figure 5 the results of such experiments with IMP dehydrogenase are shown. The results obtained with NAD and with IMP are, as expected, essentially the same and they indicate a pK value of

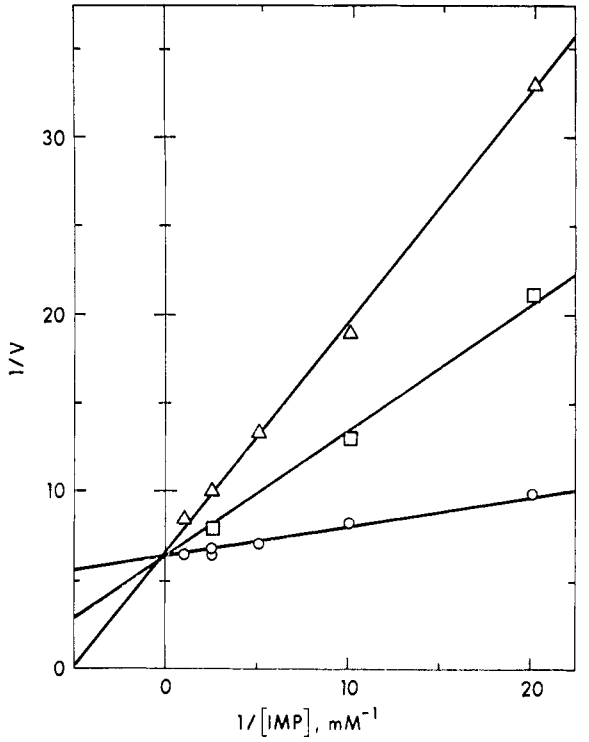

FIGURE 6: Inhibition of IMP dehydrogenase by GMP. The assay system is described in the text and contained 2.2 μg of protein. Initial velocity was measured at 350 mμ. GMP concentrations were: no GMP (○); 0.4 mM (□); 1.0 mM (△).

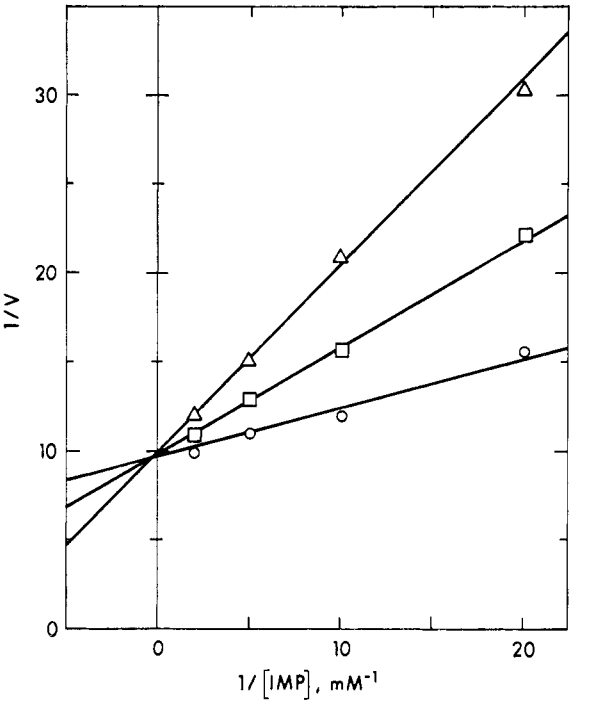

FIGURE 7: Inhibition of IMP dehydrogenase by 6-thio-IMP. Reaction conditions were the same as in Figure 6, except that 2.9 μg of protein was used. 6-Thio-IMP concentrations were: no inhibitor (○); 0.16 mM (□); 0.4 mM (△).

7.5–8.0 for an ionizing group in the enzyme–substrate complex. This could correspond to the group of pK = 8 seen in the pK_m–pH plot of NAD.

6-Thio-IMP is also a substrate of IMP dehydrogenase (Hampton, 1963). The effect of pH on the K_m of this nucleotide is shown in Figure 4. The K_m values and pH response are very similar to those of IMP, though there is a slight increase in K_m in the more acid pH region. At pH 8.1 the K_m value was 2.0×10^{-5} M.

The 6-thio-XMP formed enzymically from 6-thio-IMP has strong absorption in the 350-mμ region and the absorption coefficient varies significantly over the pH range of the present experiments. Determination of the variation of V_{max} with pH was, therefore, not possible from the above data on the variation of K_m with pH.

Inhibition by GMP and 6-Thio-IMP. The kinetic analyses shown in Figures 6 and 7 indicate that GMP and 6-thio-IMP behave as competitive inhibitors for IMP. The inhibitor constants were 1.3×10^{-4} and 1.4×10^{-4} M, respectively. GMP also shows competitive kinetics ($K_i = 3 \times 10^{-4}$ M) with IMP dehydrogenase of Sarcoma 180 tumor cells (Anderson and Sartorelli, 1966), and 6-thio-IMP ($K_i = 3.6 \times 10^{-6}$ M) behaves similarly toward IMP dehydrogenase of Ehrlich ascites cells (Atkinson *et al.*, 1963). When glutathione was omitted from the present system, 6-thio-IMP became much more inhibitory. The progressive nature of this inhibition and the protection afforded by IMP are illustrated in Figure 8. Protection of the enzyme by NAD (tested at 1 mM concentration) was not observed and, if demonstrable, would clearly be much less than that of IMP (tested at 200 μM). When the enzyme was preincubated for 10 min with 6-thio-IMP under the conditions of Figure 8 but in the absence of both IMP and NAD, 100% inactivation occurred, showing that NAD does not significantly promote the inactivation. In another experiment with the same conditions as Figure 8 the preincubation with thio-IMP was omitted, and conversion of IMP to XMP was started by addition of NAD and the thio-IMP was added 5 min later. Progressive inhibition occurred, and after 5 min this reached a value of 62% and increased only slightly thereafter.

When intermediate concentrations of glutathione were used, attempts at kinetic analysis of the inhibition by 6-thio-IMP gave nonlinear plots. In the case of IMP dehydrogenase from Ehrlich ascites cells, however, 6-thio-IMP did give linear plots and showed competitive kinetics in the presence of a small (unstated) concentration of glutathione (Atkinson *et al.*, 1963).

Inhibition by 6-Chloropurine Nucleotide. When the enzyme was preincubated with 5 μM 6-chloropurine

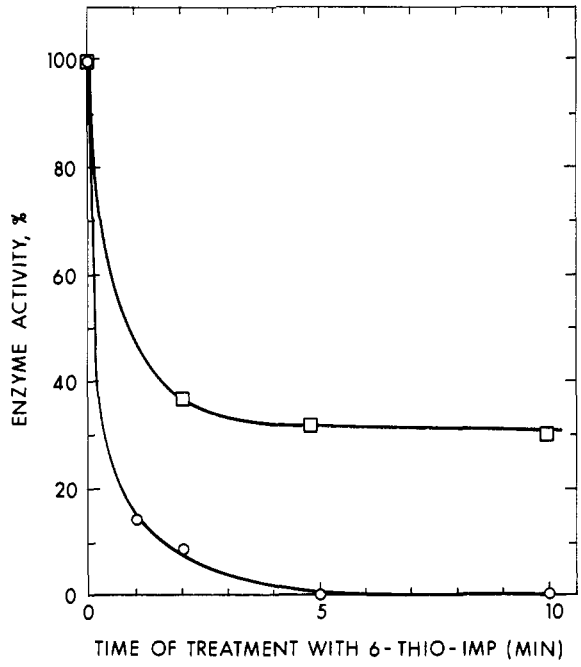

FIGURE 8: Inactivation of IMP dehydrogenase by 6-thio-IMP in the absence of glutathione. The assay system is described in the text. To a reaction mixture containing 2.2 μg of protein, 6-thio-IMP (final concentration, 1 μM) was added. After the indicated period of treatment at room temperature, the reaction was started by addition of IMP (○). In the upper curve (□), 200 μM IMP was present before the addition of 6-thio-IMP and the reaction was started by addition of NAD.

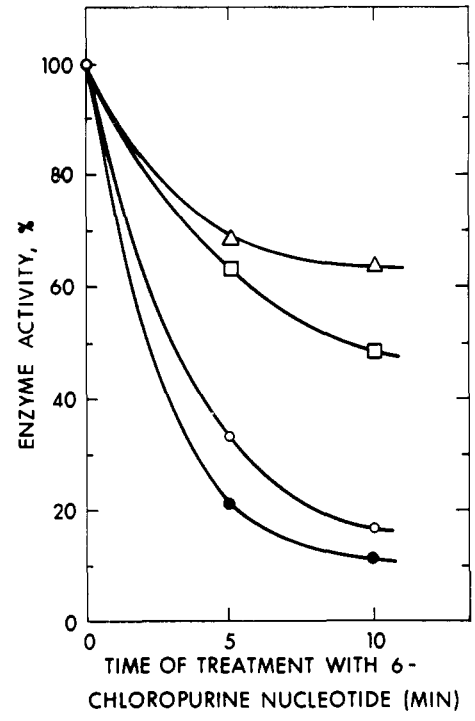

FIGURE 9: Effect of glutathione concentration on inactivation of IMP dehydrogenase by 6-chloropurine nucleotide. The system (see text) contained 4.4 μg of protein and varying amounts of glutathione. 6-Chloropurine nucleotide (final concentration, 5 μM) was added prior to the addition of enzyme. Conversion of IMP to XMP was initiated by the simultaneous addition of IMP and glutathione (final concentration, 2 mM). Glutathione concentrations during preincubation of enzyme and inhibitor were: no addition (△); 20 μM (□); 200 μM (○); 2 mM (●).

nucleotide a progressive loss in activity occurred. The extent of inactivation within a given time increased as the level of glutathione increased from 0 to 2 mM (Figure 9). The degree of inactivation tended to be maximal after 20 min (Figures 10–12). In these experiments the enzyme activity in the absence of glutathione was 26% of that in the presence of 2 mM glutathione, while the activity with 20 and 200 μM glutathione, respectively, was 59 and 97% of that with 2 mM glutathione. When glutathione and IMP were absent, preincubation of enzyme with a 10 μM solution of 6-chloropurine nucleotide for 10 min completely abolished enzymic activity in the absence of glutathione; upon addition of glutathione (400 μM), 43% of the maximal activity was manifested.

Protection of IMP Dehydrogenase by Nucleotides. IMP, GMP, and 6-thio-IMP protected the enzyme against 6-chloropurine nucleotide in the presence of glutathione. As shown in Figure 10, the order of protective efficiency is IMP > 6-thio-IMP > GMP. NAD at 1 mM did not protect the enzyme against 6-chloropurine nucleotide under these conditions, nor did it accelerate the protections or inhibitions by the above nucleotides.

In the presence of glutathione, 200 μM 6-thio-IMP was less effective than 20 μM IMP in slowing the reaction between the enzyme and 6-chloropurine nucleotide (Figure 11). In the absence of glutathione (Figure 12), 20 μM IMP was about eightfold less effective for protection of the enzyme than was 2 μM 6-thio-IMP. When the enzyme was exposed for 5 min to the action of 5 μM 6-chloropurine nucleotide in the presence of 20 μM 6-thio-IMP (Figure 12), 97% of the catalytic activity was preserved, and after continuation of this treatment for a further 15 min 98% of the activity remained. Thus, although Figure 12 shows a slow decrease in activity with 20 μM 6-thio-IMP, this decrease was paralleled in this particular experiment by a slow loss in enzyme activity which occurred in the absence of 6-chloropurine nucleotide. The rate of inactivation of IMP dehydrogenase by 6-chloropurine nucleotide under the conditions of Figure 12 was unaffected by the presence of 1 mM NAD.

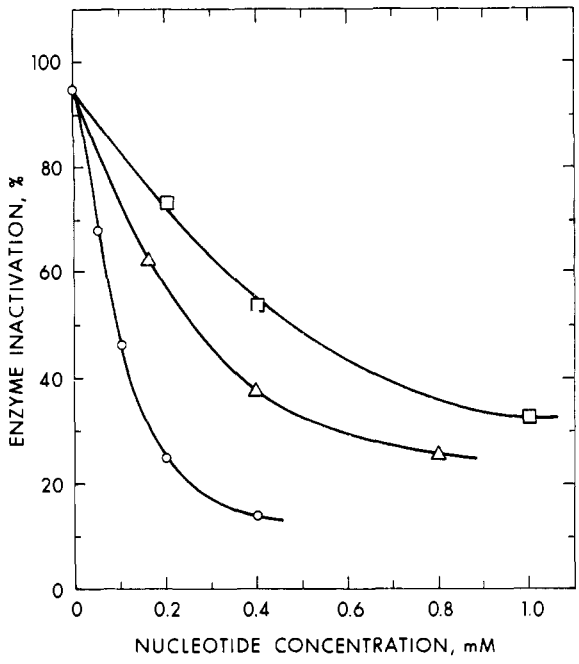

FIGURE 10: Effect of various nucleotides on the inactivation of IMP dehydrogenase by 10 μM 6-chloropurine nucleotide. The assay system is detailed in the text. 6-Chloropurine nucleotide and protector nucleotide were added before the addition of enzyme (2.9 μg of protein). After 10 min at room temperature, the reaction was started by addition of IMP. Protector nucleotides were IMP (○), 6-thio-IMP (△), and GMP (□).

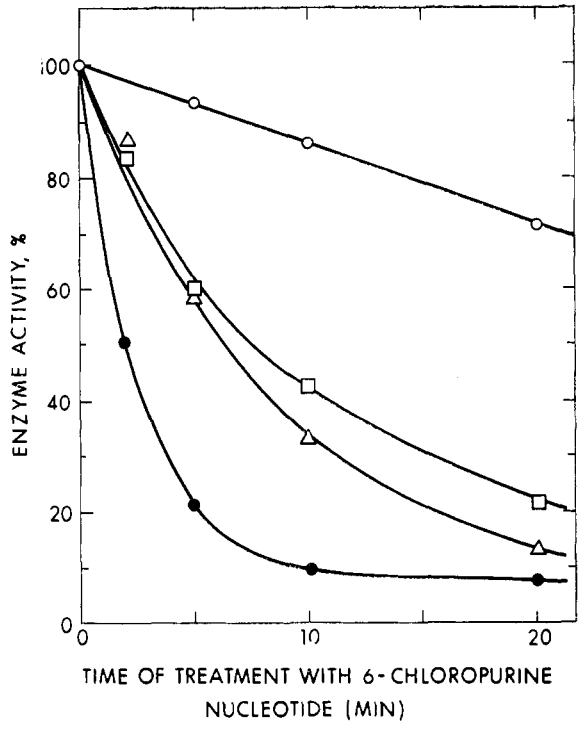

FIGURE 11: Effect of IMP and 6-thio-IMP on the inactivation of IMP dehydrogenase by 5 μM 6-chloropurine nucleotide in the presence of glutathione. The mixtures (composition given in text) contained 4.4 μg of protein. Enzymic conversion of IMP to XMP was started by addition of NAD and IMP. The concentrations of protector nucleotides present during enzyme inactivation were: no addition (●); 200 μM 6-thio-IMP (△); 20 μM IMP (□); 200 μM IMP (○). The last reaction was started by addition of NAD only.

Discussion

Previous studies (Hampton, 1963) showed that the 6-chloro and 6-mercapto analogs of IMP inactivate IMP dehydrogenase with a facility and in a manner which suggests that these analogs might be able to function as selective reagents for an amino acid or acids at the IMP site of IMP dehydrogenase. With the object of examining this possibility, economical purification procedures for the enzyme have been sought and 150-fold increase in specific activity with 70% loss of total activity has been realized (Table I). The purification included the removal of GMP reductase (Figure 1). This enzyme reacts covalently with the 6-chloro and 6-mercapto analogs of IMP about as readily and under the same conditions as does IMP dehydrogenase (Hampton *et al.*, 1966). The removal of GMP reductase is therefore a prerequisite for attempts to identify amino acids at the IMP site of IMP dehydrogenase in which partly purified IMP dehydrogenase is employed. Among other enzymes likely to similarly interfere with such attempts, adenylosuccinate synthetase (of *Escherichia coli*) and adenylosuccinate lyase (of yeast) are not inactivated by the 6-chloro and 6-thio analogs of IMP (Hampton, 1962), and GMP synthetase is not synthesized by the mutant of *A. aerogenes* used as the source of IMP dehydrogenase in this work (Moyed and Magasanik, 1957).

The above purification also freed IMP dehydrogenase from a component or components which catalyzed the hydrolysis of 6-chloropurine nucleotide and its nucleoside to products with the properties of IMP and inosine, respectively (A. Hampton and L. W. Brox, unpublished data). Adenosine deaminases from calf intestine (Cory and Suhadolnik, 1965; Frederiksen, 1966), rat heart (Baer *et al.*, 1966), and *Aspergillus oryzae* (Wolfenden, 1966) possess dechlorinase activity toward 6-chloropurine ribonucleoside. This suggests that the decomposition of 6-chloropurine nucleotide noted in the present instance might be mediated by an AMP deaminase or an adenosine deaminase.

The purified IMP dehydrogenase was characterized with respect to requirement for potassium ion, activation by glutathione, pH optimum, and Michaelis constants of IMP and NAD. No major differences were noted from the values originally recorded for a preparation of the enzyme purified by a different procedure (Magasanik *et al.*, 1957). The Michaelis constant for IMP at pH 8 (the optimum pH was 8.2) was found

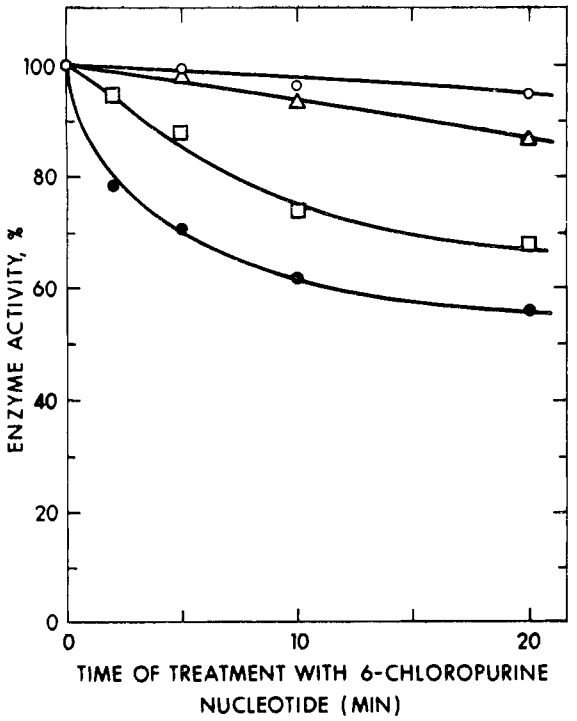

FIGURE 12: Effect of IMP and 6-thio-IMP on the inactivation of IMP dehydrogenase by 6-chloropurine nucleotide in the absence of glutathione. Conditions were the same as in Figure 11, except that glutathione was omitted during the pretreatment and added with IMP and NAD to start the reaction. Nucleotide concentrations were: no addition (●); 20 μM IMP (□); 2 μM 6-thio-IMP (△); 20 μM 6-thio-IMP (○).

to be independent of NAD concentration, while the Michaelis constant for NAD was, in turn, independent of IMP concentration (Figures 2 and 3). It has been shown (Frieden, 1957) for other two-substrate reactions that under these conditions the Michaelis constant is identical with the enzyme–substrate dissociation constant; in the present system, this would apply to both NAD and IMP. This, in turn, implies that binding of IMP and NAD to the enzyme could occur in a noncompulsory order (Frieden, 1957). In line with this possibility was the inability of NAD to affect the reaction rates of the enzyme with the 6-thio or 6-chloro analogs of IMP either in the presence or absence of glutathione, or to influence the degree to which these inactivations were slowed by various nucleotides (Figures 8–12, discussed below). However, for most dehydrogenases so far studied, an enzyme–pyridine nucleotide complex is thought to be an obligatory intermediate (Shifrin and Kaplan, 1960; Bloomfield *et al.*, 1962; Walsh and Sallach, 1965).

Binding of IMP to IMP dehydrogenase has been postulated (Hampton, 1963) to involve formation of a salt linkage between a cationic group of the enzyme and an ionic species of IMP which bears a negative charge on the 6-hydroxypurine portion. Infrared spectroscopy shows that the negative charge of the inosine anion (and hence of the above IMP anion) is principally localized on the oxygen atom at the 6 position (Miles, 1959). 6-Thio-IMP is also a substrate of the enzyme (Hampton, 1963), and the pK_a of its 6-mercapto group should be about one unit lower than the pK_a of the 6-hydroxyl of IMP, as is true for 6-thioinosine (pK_a = 7.7) and inosine (pK_a = 8.7) (Fox *et al.*, 1958). To determine if this difference in acidity could be utilized to obtain evidence as to the particular ionic species of IMP which binds to the enzyme, the effect of pH upon the Michaelis constants of IMP and 6-thio-IMP was determined (Figure 4). For both substrates a group of pK_a = 8.3 appears to be involved in the enzyme–substrate complex; since this value does not correspond to any ionizable group common to both nucleotides, it can tentatively be assigned to a group on the enzyme. Such a group could participate in binding of the phosphate moiety of the nucleotides or it might correspond to the basic group postulated to form a salt linkage with the oxygen at position 6 of IMP. The significance of the apparent difference in binding between IMP and 6-thio-IMP at relatively acidic pH values (Figure 4) is difficult to assess, particularly since no group of pK_a = 7.7 appears to be involved.

Inhibition of IMP dehydrogenase by GMP and reversal of the inhibition by IMP was reported by Mager and Magasanik (1960). Since IMP is the common precursor of AMP and GMP, the action of GMP can be viewed as a further example of feed-back inhibition of an enzymic step which immediately follows a metabolic branching point. Evidence that inhibition of IMP dehydrogenase by GMP does play a significant role in regulation of purine nucleotide interconversions has been detailed by Magasanik and Karibian (1960). In this light, IMP dehydrogenase might be expected to be subject to an allosteric type of end-product inhibition. Figure 8 shows that the inhibition by GMP is purely competitive as judged by standard double-reciprocal plots. Figure 10 shows the relative efficacies of IMP and GMP in slowing the inactivation of IMP dehydrogenase by 6-chloropurine nucleotide; IMP was between 5 and 6.5 times more effective than GMP. From the data of Figure 6 it can be calculated that the rate of conversion of IMP to XMP is reduced by 50% when the concentration of GMP is 6.5 times greater than that of IMP. The numerical coincidence of these two competitive effects argues that the action of GMP in both instances is exerted at the IMP site of the enzyme. Experiments of the same type with GMP reductase (Hampton *et al.*, 1966) have revealed that its inhibition by ATP is, by contrast, probably allosteric in nature.

6-Thio-IMP exhibited a purely competitive type of inhibition of IMP dehydrogenase when the enzyme was fully activated by glutathione (Figure 7); a sevenfold excess of 6-thio-IMP over IMP was required for 50% reduction in initial rate. Under these conditions the Michaelis constant of 6-thio-IMP is the same as that of IMP and thus the degree of inhibition by

6-thio-IMP is unexpectedly weak. Furthermore, 6-thio-IMP was three- to tenfold less effective than IMP in reducing the rate at which IMP dehydrogenase was inactivated by 6-chloropurine nucleotide (Figures 10 and 11). One explanation of these findings would be that the Michaelis constant of 6-thio-IMP is not identical with the dissociation constant of the enzyme–substrate complex, as appears to be the case with IMP.

When the glutathione concentration was reduced to levels insufficient for full enzyme activity, conversion of IMP to XMP in the presence of 6-thio-IMP became subject to a progressively increasing type of inhibition. When glutathione was absent, all enzyme activity disappeared within 5 min in the presence of 1 μM 6-thio-IMP; significant reduction in the rate of this inactivation was afforded by a 200-fold excess of IMP (Figure 8). These observations support previous lines of evidence (Hampton, 1963) that 6-thio-IMP inactivates IMP dehydrogenase by bonding to the IMP site. Also in support of this view are studies of the ability of IMP and 6-thio-IMP to retard the reaction between 6-chloropurine nucleotide and IMP dehydrogenase: in the presence of glutathione, IMP was about ten times more effective than was 6-thio-IMP (Figure 11), whereas in the absence of glutathione, 6-thio-IMP was about 80 times more effective than IMP (Figure 12). The degree to which IMP reduced the rate of inactivation during the first 5 min (Figure 11 and 12) was essentially the same in the presence of glutathione as in its absence; this is consistent with the fact that the Michaelis constant of IMP with the same enzyme preparation is independent of glutathione concentration and, in addition, is in harmony with other evidence (Hampton, 1963) which indicates that 6-chloropurine nucleotide reacts at the IMP site of the enzyme. The experiments of Figure 12 showed that 20 μM 6-thio-IMP completely protected the enzyme against inactivation by 5 μM 6-chloropurine nucleotide when the enzyme was exposed to both IMP analogs simultaneously. Under these conditions either analog alone abolishes the enzyme activity exhibited under the nonreducing conditions (*e.g.*, see Figure 8), and the experiments of Figure 12, hence, serve to confirm the conclusion (Hampton, 1963) that 6-thio-IMP and 6-chloropurine nucleotide both react at the IMP site of the enzyme.

The level of glutathione influenced to similar degrees the enzyme activity and the extent of inactivation of IMP dehydrogenase by 6-chloropurine nucleotide (Figures 9, 11, and 12). Thus, when glutathione was absent, the enzyme activity was 30% of maximum and the 6-chloropurine nucleotide abolished about 40% of the maximum enzyme activity; with 20 μM GSH, the enzyme possessed 60% of the maximum activity and the chloropurine nucleotide abolished 55% of the maximum activity. The same concentration of 6-chloropurine nucleotide rapidly deleted the enzyme activity manifested in the absence of glutathione. These observations suggest that in the absence of glutathione (or other sulfhydryl compound) IMP dehydrogenase of *A. aerogenes* exists as a mixed population of active and latently active molecules and that 6-chloropurine nucleotide reacts preferentially with those molecules which are catalytically active.

Acknowledgments

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