Inosine 5′-Phosphate Dehydrogenase. Kinetic Mechanism and Evidence for Selective Reaction of the 6-Chloro Analog of Inosine 5′-Phosphate with a Cysteine Residue at the Inosine 5′-Phosphate Site*

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ABSTRACT: A purified preparation of inosine 5′-phosphate dehydrogenase of *Aerobacter aerogenes* showed with polyacrylamide disc electrophoresis two major protein components and two minor components which possessed the dehydrogenase activity and several trace protein components which lacked that activity. Ultracentrifugation of the preparation showed two peaks with sedimentation coefficients ($s_{20,w}$) of 9.1 and 12.1 S, respectively. Product inhibition and initial velocity data were consistent with one kinetic mechanism involving two substrates and with two mechanisms which include as a third substrate the source (presumably water) of the oxygen at carbon atom 2 of xanthosine 5′-phosphate. All three of these possible mechanisms are ordered, with inosine 5′-phosphate the first substrate to bind and xanthosine 5′-phosphate the last product to leave. The inhibitor constant of xanthosine 5′-phosphate was 1.2×10^{-4} M at pH 8.1 and 24°. The rate of inactivation of inosine 5′-phosphate dehydrogenase by different levels of 6-chloropurine ribonucleoside 5′-phosphate followed saturation kinetics. The apparent dissociation constant of the 6-chloropurine nucleotide–enzyme complex at pH 7.0 and 24° was 2.6×10^{-4} M and the apparent first-order rate constant for inactivation was 0.125 sec^{-1}. Titration of the enzyme with inhibitor showed that inactivation was paralleled by the disappearance of the absorption maximum at 263 mμ of 6-chloropurine nucleotide and the appearance of a maximum at 292 mμ which in wavelength and intensity corresponded to a 6-alkylmercaptopurine nucleotide. Inosine 5′-phosphate slowed the rate of spectral change and the rate of inactivation in equal proportion. High levels of 6-chloropurine ribonucleoside caused no enzyme inactivation or spectral changes, presumably because this nucleoside, like inosine, has no affinity for the inosine 5′-phosphate site of the dehydrogenase. At pH 9 and 24° 6-chloropurine nucleoside reacted with mercaptide ion five to eight times faster than did its 5′-phosphate derivative. It is concluded that the 6-chloropurine nucleotide exclusively derivatizes cysteine residues located at the inosine 5′-phosphate sites of the dehydrogenase and that approximately 2 molar equiv inactivate the enzyme if it is assigned a molecular weight of 200,000 from the sedimentation coefficient of 9.1 S.

The 6-chloro analog of inosine 5′-phosphate (Cl-IMP)[1] is a powerful inactivator of IMP dehydrogenase of *Aerobacter aerogenes* and is of considerable interest as a potential site-specific reagent which probably derivatizes an enzymic sulfhydryl group involved in the catalytic process (Hampton, 1963; Hampton and Nomura, 1967). It was concluded that inactivation results from attachment of the analog to the IMP site because IMP and GMP (an inhibitor competitive with IMP) retard inactivation; moreover, their relative effectiveness is paralleled by their respective dissociation constants. In addition, the 6-chloro analog of inosine has virtually no effect on enzyme activity and inhibition studies show that inosine itself has no measurable affinity for the IMP site (Nichol *et al.*, 1967). Further studies of the nature and stoichiometry of the interaction between Cl-IMP and the enzyme are described in the present communication. The results strongly indicate that Cl-IMP functions as a selective reagent for the IMP site of the enzyme under the conditions which are optimum for catalytic activity. Thus, the inactivation by Cl-IMP obeys saturation kinetics and the apparent dissociation constant of the Cl-IMP–enzyme complex is of a magnitude consistent with involvement of the IMP site. The inactivation is effected by several molar equivalents of Cl-IMP and is paralleled by ultraviolet spectral changes strongly suggestive of reaction between the Cl-IMP and a cysteine residue. This reaction appears to be restricted to the IMP sites.

Product inhibition studies of the enzyme indicate that the order of addition of substrates is obligatory and that IMP is bound first. IMP dehydrogenase of mammalian origin also appears to bind IMP first (Anderson and Sartorelli, 1967). Most other dehydrogenases which have

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[1] Abbreviations and trivial names are: IMP dehydrogenase, IMP–NAD oxidoreductase (EC 1.2.1.14); Cl-IMP, 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate. See *Biochemistry 5*, 1445 (1966).

been studied also exhibit ordered substrate addition (Cleland, 1967), but IMP dehydrogenase provides the first example in which the pyridine nucleotide coenzyme is not the first substrate added. Previous findings (Nichol *et al.*, 1967) indicated that binding of IMP to IMP dehydrogenase may be accompanied by a change in enzyme conformation, and this same change might, therefore, be required for subsequent binding of NAD at the catalytic site.

Materials and Methods

Chemicals. GSH and the sodium salts of XMP and NADH were obtained from Calbiochem. NAD was obtained from Schwarz BioResearch and sodium IMP from P-L Biochemicals. Stock solutions of the pyridine coenzymes were adjusted to pH 7.0 with KOH. Other chemicals were from sources previously described (Hampton, 1963) except for Cl-IMP which was prepared by chlorination of 6-thio-IMP by the procedure of Brox and Hampton (1968).

Enzyme and Assay. The enzyme preparation used was purified to the step 4 stage described by Hampton and Nomura (1967) and had a specific activity 2.5 to 3 times greater than that reported. This increase could be due to the use of 50% more phosphocellulose relative to protein applied during the final column chromatography. Assay mixtures contained 25 μmoles of Tris-citrate buffer (pH 8.1), 2 μmoles of GSH, 100 μmoles of KCl, 2 μmoles of NAD, and 0.4 μmole of IMP in a final volume of 1.0 ml. The increase in optical density at 290 mμ was followed with a Cary Model 15 spectrophotometer in which the cell compartment was thermostated at 23–24°. Assays were started by addition of IMP; the blanks lacked IMP only. In the kinetic experiments initial velocity is the change in optical density during 10 min.

Reaction between Cl-IMP and IMP Dehydrogenase. The above phosphocellulose-purified enzyme preparation was treated at 4° with a saturated $(NH_4)_2SO_4$ solution to 65% saturation. The precipitate was collected and dissolved in 0.02 M potassium phosphate buffer (pH 7.4) containing 2 mM GSH and 100 mM KCl to give a final protein concentration of 2 mg/ml (measured by absorption at 280 mμ). Three 5-μl portions of a neutral 3.3 mM solution of sodium Cl-IMP were added at various times to 1 ml of the above protein solution. Spectra were determined with a Cary Model 15 spectrophotometer with untreated enzyme solution in the reference cuvet.

Electrophoresis. The step 4 preparation (50–100 μg of protein) was subjected to disc electrophoresis in a Canalco Model 66 apparatus in columns (diameter 5 mm, length 65 mm) of 7% polyacrylamide gel for 1.5 hr at pH 8.2 with a constant current of 3 mA/column. Protein bands were stained with aniline blue black and IMP dehydrogenase bands were stained by treatment of columns for 10–15 min with the tetrazolium reagent mixture described below followed by immediate treatment with 7% aqueous acetic acid to minimize diffusion of stain.

Cellulose acetate electrophoresis was carried out with 15–50 μg of protein on Gelman Sepraphore III strips (1 × 6.75 in.). The protein was applied to the center of the strip with a no. 00 sable brush. Electrophoresis was continued for periods as long as 2.5 hr with gradients of 25–35 V/in. Tris-maleate buffers (0.05 M) of pH 7.4 and 8.2 were employed. Protein was stained by overnight treatment with 0.02% nigrosin in 2% acetic acid. IMP dehydrogenase could be located as a blue band by applying simultaneously to each side of the electrophoretic strip a Sepraphore strip which had been soaked in the tetrazolium reagent. Maximum band to background color intensity occurred within 15 min and the electrophoretic strip was then fixed with aqueous acetic acid.

The reagent for detection of IMP dehydrogenase activity was an aqueous solution (5 ml) which contained 15 mg of NAD, 10 mg of IMP, 3 mg of GSH, 37 mg of KCl, 3 mg of *m*-nitro blue tetrazolium chloride, 0.3 mg of phenazine methosulfate, and was neutralized with 1 N KOH. The reagent was freshly prepared every 2 days.

Kinetic Studies of the Inactivation of IMP Dehydrogenase by Cl-IMP. The study was done at pH 7.0 instead of at pH 8.1 (the optimum for conversion of IMP into XMP) in order to slow the reaction to a convenient rate. IMP dehydrogenase was added to a solution (0.84 ml) at 25° containing GSH, KCl, and 0.025 M phosphate buffer (pH 7.0). After 5 min Cl-IMP was added to give the concentrations listed in Figure 5 and after various times the inactivation was stopped and the assay was started by addition of 0.16 ml of a mixture of IMP and NAD to give the normal assay concentrations of all components. Conversion of IMP into XMP was followed at 290 mμ and 25° with a Gilford spectrophotometer and recorder; the rate was linear in all determinations.

Results

Enzyme Characterization. On polyacrylamide disc electrophoresis more than 80% of the total stained protein from the enzyme preparation moved as two bands which both stained positively with the IMP-dependent dehydrogenase reagent. With 7% polyacrylamide the mobility of the slower band was 50–60% that of the faster. Also present were four trace protein bands with no dehydrogenase activity which moved faster than the main enzyme bands and two minor bands which did show the enzyme activity and which moved slower than the main enzyme bands.

Cellulose acetate strip electrophoresis at both pH values employed showed one protein band which migrated several centimeters and which coincided with a single band of IMP dehydrogenase activity.

The phosphocellulose-purified enzyme fractions (in 0.02 M potassium phosphate and 0.25 M KCl) with the highest specific activity were centrifuged at 8° at 59,780 rpm in the Spinco Model E analytical ultracentrifuge. Two peaks were seen with $s_{20,w}$ values of 9.1 and 12.1 S at a protein concentration of 0.2%.

Product Inhibition. Figure 1 (frame A) shows that XMP gives competitive inhibition when IMP is varied with NAD at a fixed unsaturating concentration. A plot of slopes against XMP concentration (Figure 1, frame B) indicates that the inhibition is linear with a K_i of 1.2×10^{-4} M.

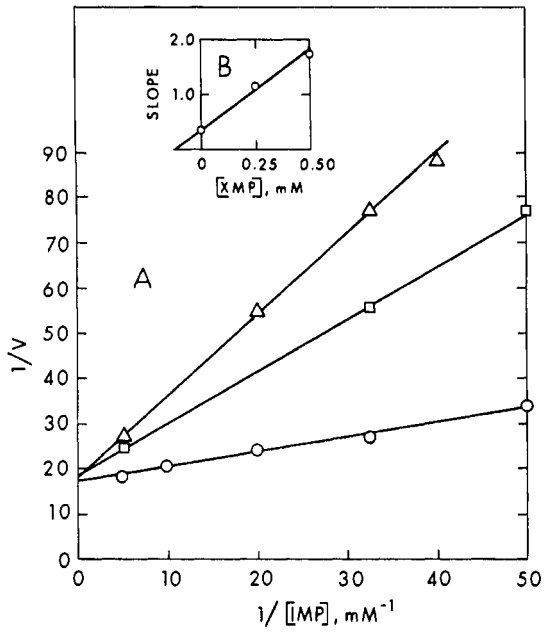


FIGURE 1: Frame A: reciprocal plot with IMP as variable substrate and XMP as inhibitor. NAD was 0.5 mM. Initial velocity is expressed as change in optical density at 290 mμ/10 min. XMP concentrations were: 0 mM (○), 0.25 mM (□), and 0.50 mM (△). Frame B: secondary plot of slopes from frame A against XMP concentration.

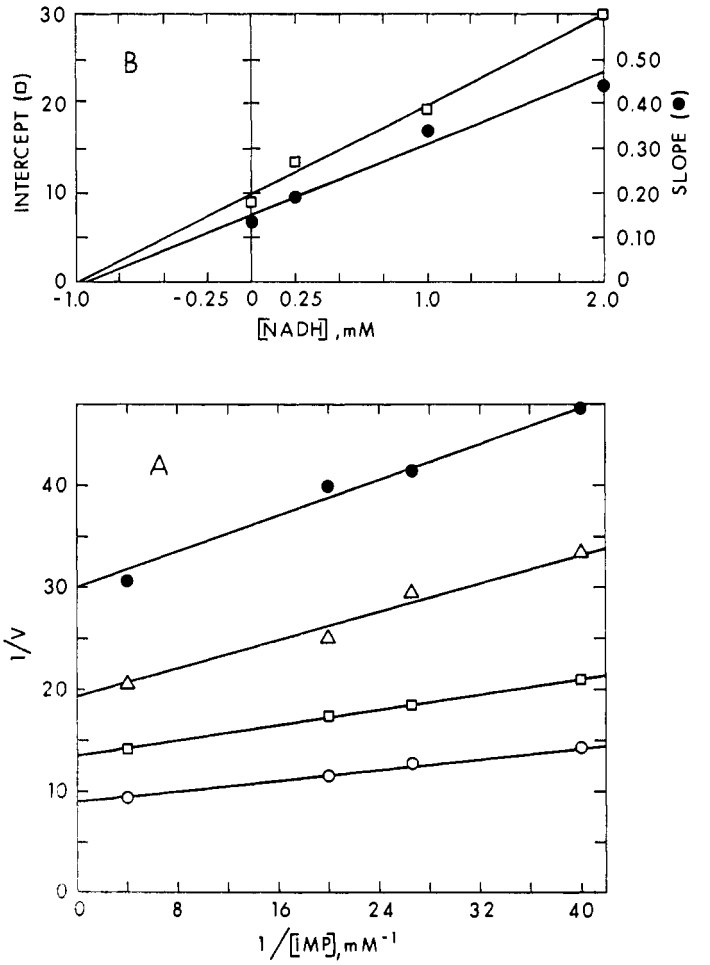


FIGURE 2: Frame A: reciprocal plot with IMP as variable substrate and NADH as inhibitor. NAD was 0.5 mM. NADH concentrations were: 0 mM (○), 0.25 mM (□), 1 mM (△), and 2 mM (●). Frame B: secondary plots of slopes and intercepts from frame A against NADH concentration.

Figure 2 shows that NADH inhibition is noncompetitive when IMP is varied and NAD is maintained at a constant unsaturating concentration. Replots of slopes and intercepts are linear functions of NADH concentration; K_i (slope) and K_i (intercept) are both about 1.0×10^{-3} M.

XMP is a noncompetitive inhibitor with respect to NAD when IMP is unsaturating (Figure 3). Inhibitions are linear with a K_i (slope) of 2.4×10^{-4} M and a K_i (intercept) of 3.8×10^{-4} M.

With a high fixed IMP concentration, NADH gives noncompetitive kinetics when NAD is varied (Figure 4). The slopes are a linear function of NADH concentration with a K_i (slope) of 1.1×10^{-3} M. The intercepts, however, are a more complex function of NADH concentration. The curve obtained (Figure 4) does not appear to be parabolic since a plot of tangents to this curve against NADH concentration is not linear.

All lines in Figures 1–4 were fitted by the method of moments (Kenney and Keeping, 1939) assuming equal weights for all points.

Kinetics of Inactivation by Cl-IMP. In Figure 5 the logarithm of the ratio of initial enzyme activity to final activity is plotted against the time (in minutes) of preincubation for different concentrations of Cl-IMP. The apparent first-order rate constants, k_{obsd}, for enzyme inactivation (in reciprocal seconds) are obtained by dividing the slopes of the plots by 60. The reciprocals of the k_{obsd} values are plotted against the reciprocal of the Cl-IMP concentration in Figure 6. The linear plot obtained was fitted by the method of moments used for Figures 1–4. The intercept on the vertical axis is 8 sec and that on the horizontal axis is 3.8×10^3 M^{-1} from which $k_2 = 0.125$ sec^{-1} and $K = 0.26 \times 10^{-3}$ M by the equation given in the Discussion.

Reaction between Cl-IMP and IMP Dehydrogenase. As shown in Figure 7, interaction of Cl-IMP (absorption maximum, 263 mμ) with purified IMP dehydrogenase (see Methods) resulted in the progressive development of a new maximum at about 290 mμ. Develop-

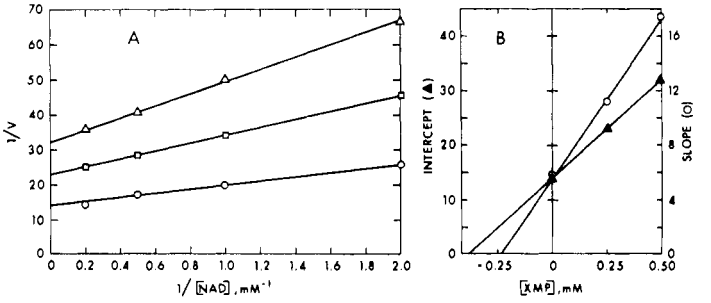


FIGURE 3: Frame A: reciprocal plot with NAD as variable substrate and XMP as inhibitor. IMP was 0.05 mM. XMP concentrations were: 0 mM (○), 0.25 mM (□), and 0.50 mM (△). Frame B: secondary plots of slopes and intercepts from frame A against XMP concentration.

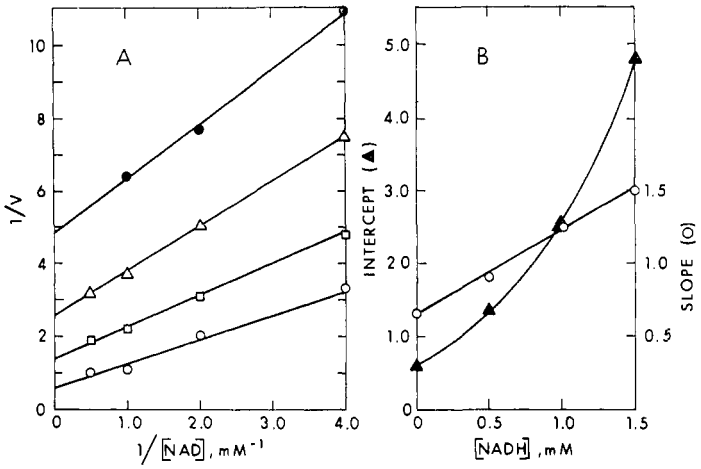


FIGURE 4: Frame A: reciprocal plot with NAD as the variable substrate and NADH as inhibitor. IMP was 0.25 mM. NADH concentrations were: 0 mM (○), 0.5 mM (□), 1 mM (△), and 1.5 mM (●). Frame B: secondary plots of slopes and intercepts from frame A against NADH concentration.

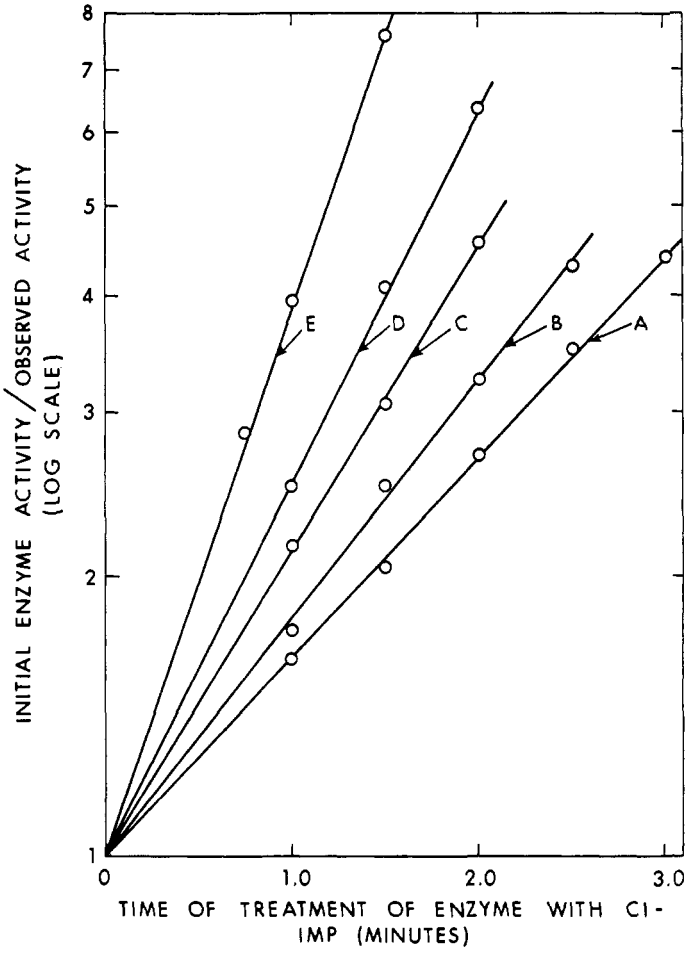


FIGURE 5: Rate of inactivation of IMP dehydrogenase by Cl-IMP at 25° and pH 7. For details, see Methods. Each point is the average of at least two determinations. Before addition of Cl-IMP the reaction velocity (change in absorbancy at 290 mμ) was 0.69 in 10 min. Concentrations of Cl-IMP: plot A, 18.4 μM; plot B, 23.0 μM; plot C, 27.6 μM; plot D, 36.8 μM; and plot E, 61.3 μM.

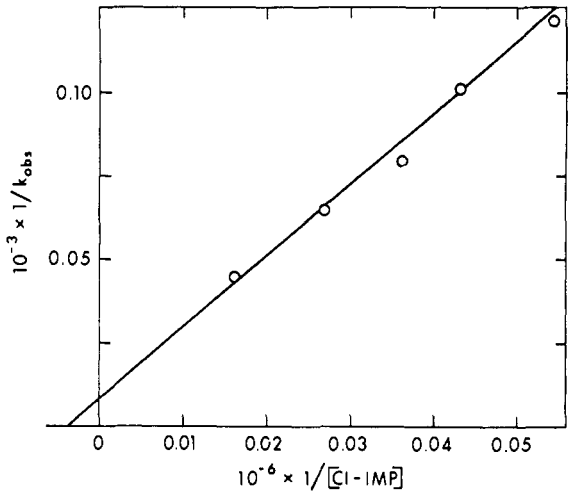


FIGURE 6: Reciprocal plot of molar concentration of Cl-IMP *vs.* the observed first-order rate constants, k_{obsd}, for irreversible inactivation. The k_{obsd} values (sec^{-1}) were calculated from Figure 5.

ment of this peak was accompanied by loss of enzyme activity (Figure 8). The absorption at 290 mμ did not tend to increase when the enzyme was omitted. Substitution of 200 μM 6-chloropurine ribonucleoside for the Cl-IMP (initially at 16.5 μM; Figure 7A) resulted in no increase in absorption at 290 mμ and no loss of activity during 2 hr. In the presence of 200 μM IMP the increase in absorption at 290 mμ brought about by 16.5 μM Cl-IMP was 0.03 with 10% inactivation of the enzyme after 20 min and 0.12 with 50% inactivation after 90 min. In the absence of IMP (Figure 7A) 50% inactivation occurred after approximately 18 min.

The molar absorptivity of a 6-alkylmercaptopurine nucleotide formed from the reaction of Cl-IMP with IMP dehydrogenase presumably has a value of about 16,800 since for 6-methylmercaptopurine ribonucleoside ε is 16,400 (Montgomery *et al.*, 1961) and for 6-succinomercaptopurine ribonucleotide (pH 7.4–9) ε is 17,200 at λ_{max} 291 mμ (Hampton, 1962). On this assumption the optical density value of 0.37 at 292 mμ which is associated with deletion of 95% of enzyme activity (Figure 8) would correspond to formation of 22 μM 6-alkylmercaptopurine nucleotide. The concentration of IMP dehydrogenase in the same mixture would be 10 μM if the preparation was completely homogeneous and had a molecular weight of 200,000. The observed optical density values at 292 mμ did not require correction for the absorption of Cl-IMP (ε 180 at 290 mμ, pH 7.9) at that wavelength.

If the above conclusions are correct, then the twin-peaked absorption spectrum obtained 30 min after the final addition of Cl-IMP (Figure 7B) should resemble that of a mixture of 28 μM Cl-IMP and 22 μM 6-alkylmercaptopurine nucleotide. At 263 mμ the ε value for Cl-IMP is 8400 at the pH of these studies (Hampton and Maguire, 1961) and a 6-alkylmercaptopurine nucleotide would have an ε value of *ca.* 5200 (calculated from data of Hampton, 1962) at the same pH. In the above mixture of the two nucleotides the ratio of optical density at 290 mμ to optical density at 263 mμ should be *ca.*

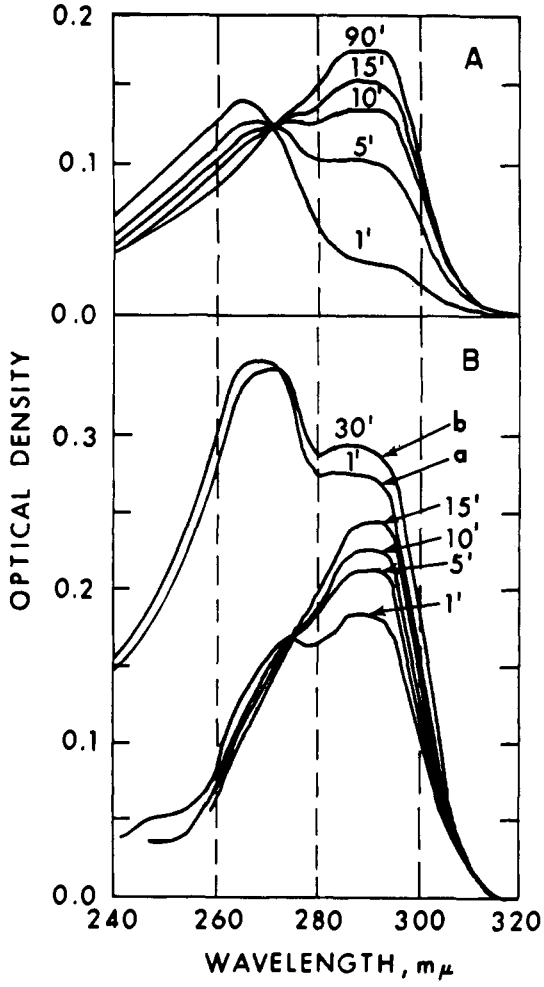


FIGURE 7: Spectral changes resulting from reaction of Cl-IMP with IMP dehydrogenase. Cl-IMP (16.5 mμmoles) was added to *ca.* 10 mμmoles of the enzyme under conditions described in Methods (frame A); a further 16.5 mμmoles of Cl-IMP was added after 100 min (frame B) and after a further 20 min (frame B, curves a and b).

1.06; the value obtained in the experiment of Figure 7 is 0.93.

Relative Reactivity of Cl-IMP and Its Nucleoside toward Aliphatic Mercaptans. Figure 9 shows the rate of increase in optical density at 290 mμ when GSH or β-mercaptoethanol is allowed to react nonenzymatically at pH 9.0 with Cl-IMP or 6-chloropurine ribonucleoside. After sufficiently long reaction times the absorption maximum at 263 mμ of the 6-chloropurine nucleoside was replaced by a new maximum at 292 mμ characteristic of a 6-alkylmercaptopurine nucleoside at slightly alkaline pH (Hampton, 1962). The 6-chloropurine nucleoside reacted five times faster than Cl-IMP in bicarbonate buffer and 8.5 times faster in Tris-HCl buffer.

Discussion

The formation of XMP from IMP requires, in addition to the pyridine coenzyme, a source of the oxygen at C-2 of XMP. This oxygen source (presumably some form of a water molecule) might bind to an enzymatic

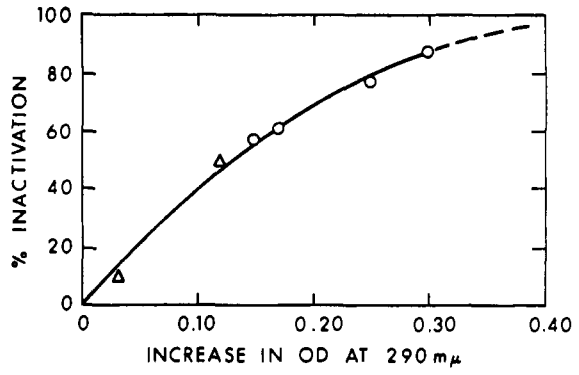


FIGURE 8: Inactivation of IMP dehydrogenase by Cl-IMP as a function of increase in absorption at 290 mμ. Values presented as open circles are from the experiment of Figure 7 and values presented as open triangles are from a parallel experiment carried out in the presence of 200 μM IMP and (initially) 16.5 μM Cl-IMP.

site prior to reaction and thus function kinetically as a third substrate, or it might react directly with an activated enzyme–substrate complex. The latter mechanism could result in a two-substrate–two-product system; for such a system the initial velocity and product inhibition data are consistent with an ordered kinetic model (A) with sequential addition of IMP and NAD and sequential release of NADH and XMP. Table I presents for model A the various apparent inhibition constants,

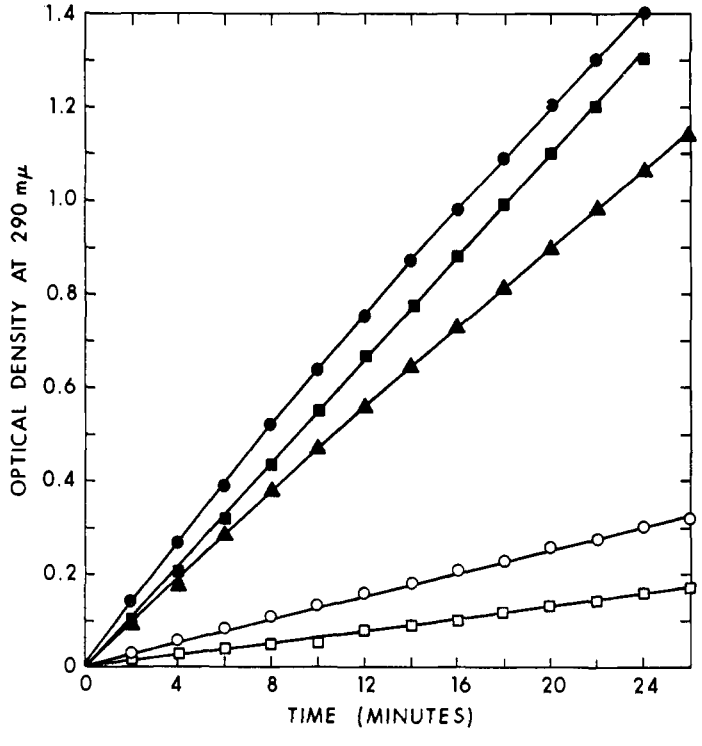
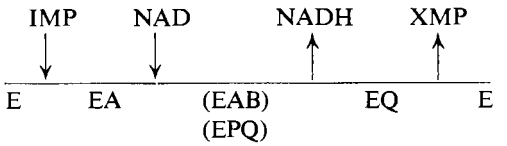


FIGURE 9: Rates of nucleophilic attack on carbon atom 6 of Cl-IMP and of 6-chloropurine ribonucleoside by aliphatic mercaptans. All reactants are 1 mM and buffers 0.1 M pH 9.0; temperature, 24°. Nucleoside and GSH in sodium bicarbonate buffer (●); nucleotide and GSH in bicarbonate (○); nucleoside and GSH in Tris-HCl (■); nucleotide and GSH or β-mercaptoethanol in Tris-HCl (□); and nucleoside and β-mercaptoethanol in Tris-HCl (▲).

TABLE I: Kinetic Expressions Calculated for Model A for Apparent Inhibition Constants, K_i, of Products.[a]

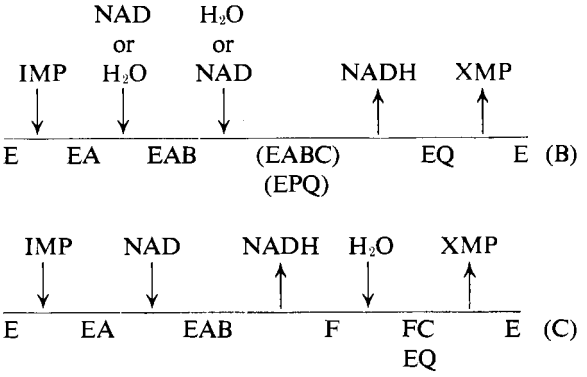
Substrate	Inhibitor	Expressions for K_i Values	Obsd K_i Values (M)
IMP	XMP	K_i (slope) $= K_{iQ}$	1.2×10^{-4}
NAD	XMP	K_i (slope) $= \frac{K_{iQ}(K_{iA} + A)}{K_{iA}}$	2.4×10^{-4}
		K_i (intercept) $= \frac{K_{iQ}(K_A + A)}{K_A}$	3.8×10^{-4}
IMP	NADH	K_i (slope) $= (K_P K_{iQ}/K_Q)\left(1 + \frac{K_A B}{K_{iA} K_B}\right)$	1.0×10^{-3}
		K_i (intercept) $= \frac{K_P K_{iP} K_{iQ}(K_B + B)}{K_B K_P K_{iP} + K_P K_{iQ} B}$	1.0×10^{-3}
NAD	NADH	K_i (slope) $= \frac{K_P K_{iQ}}{K_Q}$	1.1×10^{-3}
		K_i (intercept) $= K_{iP}\left(\frac{K_A + A}{A}\right)$	*b*

[a] The terminology of Cleland (1963a,b) is employed in which K_A, K_B, K_P, and K_Q are the Michaelis constants of IMP, NAD, NADH, and XMP, respectively, and K_{iA}, K_{iB}, etc., are termed inhibition constants. [b] Replot not linear.



K_i's, from product inhibition experiments in terms of kinetic constants and substrate concentrations, together with the numerical values obtained. In this kinetic model the inhibition constants K_{iA} and K_{iQ} are simple dissociation constants. From the first three expressions in Table I the dissociation constant for XMP, K_{iQ}, is calculated to be 1.2, 0.8, and 1.3×10^{-4} M, respectively (average, 1.1×10^{-4} M), by assuming that $K_A = K_{iA} = 0.21 \times 10^{-4}$ M as concluded previously from initial velocity studies (Hampton and Nomura, 1967). This indicates that IMP binds to IMP dehydrogenase about five times stronger than does XMP. The remaining Michaelis and inhibition constants for model A could not be determined by initial velocity and product inhibition studies because the equilibrium position of the conversion was too much in favor of XMP formation to permit study of the reverse reaction by the present spectrophotometric method.

If the oxygen source for carbon atom 2 of XMP is treated as a third substrate then kinetic constants analogous to those of Table I cannot be calculated. The initial velocity and product inhibition data would be consistent with two ordered kinetic models (B and C) provided that the oxygen source is not saturating. Saturation by the oxygen source appears to eliminate all possible kinetic models for the system with three substrates. For example, in the case of model B saturation by water would convert otherwise noncompetitive product inhibitions into uncompetitive ones, and for model C such saturation would drive the NADH inhibition to zero.



IMP dehydrogenase provides the first instance among dehydrogenases of ordered addition in which the first substrate added is not the pyridine nucleotide coenzyme. Dehydrogenases believed to operate by initial addition of pyridine nucleotide coenzyme include malate dehydrogenase (Raval and Wolfe, 1962), NAD–isocitrate dehydrogenase (Sanwal *et al.*, 1965; Sanwal and Stachow, 1965), glutamate dehydrogenase (Fahien *et al.*, 1965), malic enzyme (Cleland, 1967), and yeast and liver alcohol dehydrogenase (Cleland, 1967; Hollis, 1967). Liver alcohol dehydrogenase can function to a limited extent by random addition of substrates (Cleland, 1967), while in the case of glyceraldehyde 3-phosphate dehydrogenase (Furfine and Velick, 1965) random addition appears to predominate. Studies of the structural requirements for binding to the IMP site of IMP dehydrogenase suggested that IMP binding requires a con-

figurational modification mediated partly by interaction of the phosphate moiety of IMP with the enzyme (Nichol *et al.*, 1967). It is conceivable that such configurational transitions might be a prerequisite for specific binding of NAD to IMP dehydrogenase.

Models A and B could be interpreted mechanistically as addition of water across the 2,3 double bond of IMP associated with transfer of the C-2 hydrogen to NAD. An elaboration of this over-all mechanism has been previously presented (Hampton, 1963) and postulates activation of C-2 for attack by hydroxyl ion by addition of an enzymic sulfhydryl group across the 1,6 double bond of IMP. The oxygen source, NAD, and IMP are considered to be present simultaneously on the enzyme so as to permit the catalytic process to proceed in concerted fashion. Model C, on the other hand, implies that an enzyme–IMP derivative is formed during transfer of the C-2 hydrogen to NAD. Such a mechanism resembles that of glyceraldehyde 3-phosphate dehydrogenase in which an enzymic sulfhydryl group forms an *S*-acyl derivative of glyceric acid 3-phosphate during transfer of the aldehyde hydrogen atom to NAD (Krimsky and Racker, 1955). By analogy, an enzymic group of IMP dehydrogenase could form a bond at C-2 during transfer of the C-2 hydrogen to NAD and the resulting enzyme–IMP derivative could react with the oxygen source to produce XMP and regenerate the enzyme.

IMP dehydrogenase is rapidly inactivated by low concentrations of the 6-chloro analog of IMP (Cl-IMP) and evidence presented previously (Hampton, 1963; Hampton and Nomura, 1967) indicated that this process occurs at the IMP site. The effect of Cl-IMP concentration upon the rate of inactivation was studied (Figures 5 and 6); the saturation effect (Baker *et al.*, 1962) which was observed confirms that inactivation by Cl-IMP is preceeded by a reversible binding to the enzyme (eq 1)

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E\cdot I \xrightarrow{k_2} E\cdot I \text{ (inactive)} \qquad (1)$$

and is not simply random bimolecular. Gold and Fahrney (1964) developed a kinetic expression (eq 2) for

$$1/k = 1/k_2 + (K/k_2)(1/[I]) \qquad (2)$$

for such two-stage inactivations in which k is the observed first-order rate constant for inactivation, k_2 is the first-order rate constant for the actual inactivation step, K is $(k_{-1} + k_2)/k_1$ (see eq 1), and [I] is the inhibitor concentration. Equation 2 has been used to determine k_2 and K for irreversible inactivations of adenosine deaminase (Schaeffer *et al.*, 1967) and 2-keto-3-deoxy-6-phosphogluconic aldolase (Meloche, 1967). If k_{-1} is much greater than k_2, then K closely approximates to the inhibitor–enzyme dissociation constant and this equivalence has been demonstrated experimentally for site-specific alkylations of adenosine deaminase (Schaeffer *et al.*, 1967). With Cl-IMP and IMP dehydrogenase the value of K at pH 7 was 2.6×10^{-4} M, and this could well correspond to the dissociation constant for Cl-IMP because it is of the same order as the dissociation constants at pH 8 (1.3 and 3.1×10^{-4} M) of GMP and 6-thio-IMP, respectively.

Previous studies (Hampton, 1963) provided indirect evidence that Cl-IMP may inactivate IMP dehydrogenase by forming a thioether bond between C-6 of the purine ring and a sulfhydryl group at the IMP site. Strong support for this postulate is provided by the spectral changes (Figure 7) which accompany reaction of approximately equimolar amounts of IMP dehydrogenase and Cl-IMP and which terminate when enzyme inactivation reaches completion. The absorption maximum due to Cl-IMP diminishes and a new peak at 292 mμ simultaneously appears. Among 6-substituted 9-alkylpurines (such as Cl-IMP) an absorption maximum at pH 8 of 292 mμ is characteristic of 6-alkylmercapto derivatives, *e.g.*, 6-methylmercapto-9-β-D-ribofuranosylpurine (Montgomery *et al.*, 1961); by contrast, at the same pH, 6-alkylamino derivatives such as 6-methylamino have maxima at 266–267 mμ, and 6-alkoxy derivatives have maxima at 248 mμ (Johnson *et al.*, 1958). Additional evidence for formation of a 6-alkylmercaptopurine nucleotide is afforded by the intensity of the absorption maximum at 292 mμ which agrees with that expected from the known extinction coefficient of such derivatives.

IMP slowed the enzyme inactivation by Cl-IMP and the formation of a 6-alkylmercaptopurine nucleotide to equal extents. Further evidence that the site of formation of the 6-alkylmercapto derivative is the IMP site was afforded by the finding that 6-chloropurine ribonucleoside caused no inactivation and no absorbancy increases at 292 mμ for prolonged periods of time when present at 12-fold higher concentration than Cl-IMP. The inertness of the nucleoside of Cl-IMP cannot be ascribed to relatively low electrophilicity of its 6 carbon, because under conditions similar to those of enzyme inactivation the nucleoside of Cl-IMP is attacked at C-6 by aliphatic mercaptide ion five to ten times faster than is Cl-IMP itself (Figure 9). The nucleoside of 6-chloropurine, like inosine (Nichol *et al.*, 1967), is probably unable to induce the conformational change in the enzyme which appears to accompany binding of IMP to its site.

The relationship between enzyme inactivation and formation of a 6-alkylmercaptopurine nucleotide derivative of the enzyme lacked complete linearity (Figure 8), particularly at the higher levels of inactivation. This is unlikely to be due to combination of Cl-IMP with sulfhydryl groups not at the active site because 6-chloropurine nucleoside could also be expected to react with such groups yet it does not. The nonlinearity might result from the existence of sites with differing degrees of catalytic activity and preferential reaction of Cl-IMP with the most active of these sites. The present estimate of the molecular weight (*ca.* 200,000) of the enzyme indicates that it could become inactivated after reaction with as little as 2 molar equiv of Cl-IMP.

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Reactivity of the Sulfhydryl Groups of Muscle Phosphofructokinase*

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ABSTRACT: Rabbit skeletal muscle phosphofructokinase contains 16.9 ± 0.3 cysteinyl residues per 90,000 g of enzyme. On the basis of their reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid), these cysteinyl residues may be classified into five groups. A single exceedingly reactive SH group exhibits an apparent second-order rate constant that is 2×10^4 times higher than the rate of the reaction of denatured phosphofructokinase with the disulfide compound. The second class of SH groups consists of two cysteinyl residues that are somewhat less reactive and are protected from reaction by either adenine nucleotides or fructose 6-phosphate. The reaction of these two SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) results in the loss of more than 90% of the enzyme activity. The third class consists of one SH group that reacts at about 0.4 of the rate of the second class. Approximately five SH groups become available for rapid reaction with 5,5′-dithiobis(2-nitrobenzoic acid) as the pH of the reaction medium is increased to 9. Two of these SH groups are protected from reaction with 5,5′-dithiobis(2-nitrobenzoic acid) by fructose 1,6-diphosphate.

The remaining SH groups react very slowly with 5,5′-dithiobis(2-nitrobenzoic acid) and can be made available for rapid reaction only if the tertiary structure of the enzyme is destroyed. A simplified procedure for the preparation of crystalline rabbit skeletal muscle phosphofructokinase is described.

Work in recent years had indicated that phosphofructokinase represents a sensitive control point in glycolysis (Lowry *et al.*, 1964) and that its activity is regulated by interaction with a number of metabolites (Lowry and Passonneau, 1966; Kemp and Krebs, 1967).

The successful preparation of highly purified skeletal muscle phosphofructokinase has been reported by a number of laboratories (Ling *et al.*, 1965; Uyeda and Racker, 1965; Parmeggiani *et al.*, 1966). These workers have indicated that the purified enzyme is not particularly stable but that it can be readily protected from inactivation by the presence of substrates. It was early recognized that phosphofructokinase is extremely sensitive to agents that destroy thiol groups (Engel'hardt and

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