Analogs of Inosine 5′-Phosphate with Phosphorus–Nitrogen and Phosphorus–Sulfur Bonds. Binding and Kinetic Studies with Inosine 5′-Phosphate Dehydrogenase*

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ABSTRACT: Inosine 5′-phosphorothioate, 5′-mercapto-5′-deoxyinosine 5′-*S*-phosphate, and 5′-amino-5′-deoxyinosine 5′-*N*-phosphate have been synthesized and found to be substrates of inosine 5′-phosphate dehydrogenase of *Aerobacter aerogenes*. Initial velocity data at the pH optimum (8.1) was consistent with an ordered kinetic model, presumably of the BiBi type which is consistent with kinetic data when inosine 5′-phosphate is substrate, and in which inosine 5′-phosphate is the first substrate added. On this basis, enzyme–substrate dissociation constants of 23×10^{-5}, 1.7×10^{-5}, and 0.5×10^{-5} M, respectively, were determined for the above nucleotide analogs (*cf.* inosine 5′-phosphate, 2.0×10^{-5} M). The Michaelis constants were 21×10^{-5}, 1.3×10^{-5}, and 3.8×10^{-5} M, and the V_{max} values relative to that for inosine 5′-phosphate ($V_{max} = 1$) were 1.05, 0.75, and 0.67, respectively. The kinetic properties of inosine 5′-phosphorothioate can be ascribed entirely to a tenfold reduction in the rate constant for interaction of substrate and enzyme which accompanies this modification of the inosine 5′-phosphate structure. With the 5′-*S*-phosphate analog, the rate constants for enzyme–substrate association or dissociation were the same as those of inosine 5′-phosphate; with the 5′-*N*-phosphate, a reduction in both rate constants is observed. The C-5′ oxygen of inosine 5′-phosphate is concluded not to contribute significantly to the total binding energy.

The present studies appear to favor the view that inosine 5′-phosphate binds preferentially in its phosphodianion form. Ultraviolet optical rotatory dispersion determinations in aqueous solution provided no evidence for major conformational differences between the analogs and inosine 5′-phosphate itself. At pH 6 the kinetic mechanism was the same as at pH 8.1; changes in the rate constants for enzyme–substrate interaction of inosine 5′-phosphate and its 5′-*N*-phosphate analog at this pH are ascribed to changes in the enzyme.

In previous studies (Nichol *et al.*, 1967) on binding of the phosphomonoester portion of IMP to IMP dehydrogenase (IMP:NAD oxidoreductase, EC 1.2.1.14) we found that replacement of one phosphate hydroxyl of IMP by hydrogen or other small substituents eliminated affinity for the IMP site as evidenced by lack of either substrate or inhibitory properties at high concentrations. In the present report we describe the synthesis of three analogs of IMP (Figure 1) in which sulfur replaces a nonesterified phosphate oxygen and sulfur and nitrogen, respectively, replace the esterified phosphate oxygen. The analogs are substrates for IMP dehydrogenase of *Aerobacter aerogenes;* initial velocity data have been utilized to determine enzyme–substrate dissociation constants and other kinetic parameters. The effects of the changes in IMP structure on the rate constants for addition to and release from the enzyme are evaluated from the above kinetic parameters in the light of a kinetic model previously advanced for this enzyme (Brox and Hampton, 1968).

Materials and Methods

Enzyme and Assay. Chemicals for assay and kinetic studies of IMP dehydrogenase were obtained from the sources used in previous work (Brox and Hampton, 1968). The analogs of IMP were converted into potassium salts by passage through Dowex 50 (K^+) ion-exchange resin and the solutions were adjusted, if necessary, to pH 7.5–8.0 with ammonia.

The IMP dehydrogenase employed was a highly purified preparation from *A. aerogenes* obtained by phosphocellulose column chromatography (Brox and Hampton, 1968). Assay conditions were as described previously except that the Tris-citrate buffer was 0.05 M. Studies at pH 6.0 employed 2-(*N*-morpholino)ethanesulfonic acid buffer (Good *et al.*, 1966) at a final concentration of 0.05 M. Reaction was started by addition of IMP and the increase in optical density due to formation of NADH was monitored at 340 mμ and 24° using a Cary Model 15 spectrophotometer with a full-scale chart deflection of 0.10 optical density; all rates were linear with time and with enzyme concentration. The purified enzyme preparation was diluted daily with 0.01 M Tris-citrate buffer (pH 8.1) which contained glutathione (0.002 M) and KCl (0.1 M). Between 0.5 and 1.0 μg of protein from this stock solution was normally used for pH 8.1 reactions and 50–75 μg for pH 6.0

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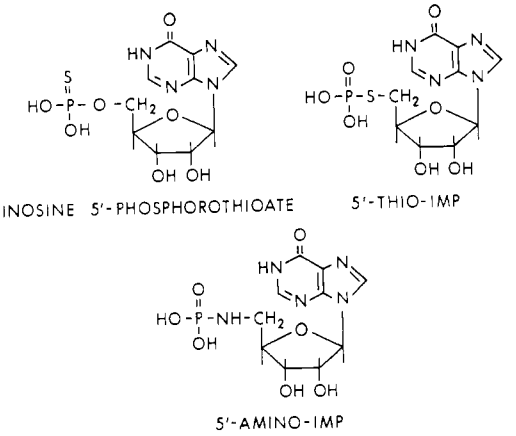


FIGURE 1: Structures of IMP analogs.

reactions. The rates at pH 6.0 were proportional to protein concentration from 25 to at least 125 μg of protein per assay.

When the data appeared to describe a linear relationship it was fitted to a straight line by the method of least squares assuming equal weighting for all points.

Physicochemical Properties and Synthesis of the IMP Analogs. The pK_a values for the secondary phosphate ionizations of IMP and its analogs were determined at 22° by potentiometric titration (Corning Model 12 pH meter) in which HCl was added to 5–8 mM aqueous solutions of the nucleotide potassium salts; for titration of 5'-amino-IMP,[1] KCl (0.1 M) was included in order to approximate more closely to the enzyme assay conditions.

Optical rotatory dispersion curves were determined with solutions of optical density at 250 mμ less than 2 in 1-cm cells in a Cary Model 60 spectropolarimeter. In cases where the specific rotation was small at 280–320 mμ, the concentration was increased twofold. Paper chromatography was carried out by the ascending method in the following systems: solvent A, 2-propanol–NH_4OH–water (7:1:2, v/v); solvent B, saturated $(NH_4)_2SO_4$–0.1 M sodium acetate (pH 6)–2-propanol (79:19:2, v/v); solvent C, isoamyl alcohol–5% aqueous Na_2HPO_4 (1:1, v/v); solvent D, 1-butanol–acetic acid–water (5:2:3,v/v); and solvent E, solvent B adjusted to pH 8 with NH_4OH. Buffers used for paper electrophoresis (30 V/cm) were 0.04 M phosphate (pH 8), 0.05 M sodium tetraborate (pH 9.2), and 0.05 M ammonium formate (pH 3.5, 6.2 and 8). Phosphate was detected on chromatograms and after electrophoresis with a molybdate–perchloric acid spray (Hanes and Isherwood, 1949) and *cis*-glycol systems were located by a periodate–Schiff's reagent spray (Baddiley and Buchanan, 1956). Other methods for chemical syntheses were the same as described previously (Nichol *et al.*, 1967).

Results

Inosine 5'-O-Phosphorothioate. 2',3'-*O*-Isopropylideneinosine (0.3 g) was added to a stirred mixture of 1 ml of acetonitrile (Fisher Certified, contained 0.1% H_2O), 0.07 ml of dry pyridine, and 0.3 ml of $PSCl_3$ at 0–5°. After 6 hr it was poured into 60 ml of ice and water, the mixture was brought to pH 2 with $Ba(OH)_2$, stirred until gum dissolved, and the solution was kept at 70–80° for 1 hr to remove the isopropylidene group. The pH was adjusted to 8 with $Ba(OH)_2$ and the precipitate was centrifuged. The product was precipitated by addition of two volumes of ethanol to the supernatant. After two more such precipitations 0.10 g (19% yield) of barium nucleotide was obtained. Elemental analysis indicated that it contained small amounts of barium phosphorothioate and this was confirmed by paper electrophoresis at pH 6.2 and application of the spray reagent for phosphate which revealed traces of a nonultraviolet-absorbing component which had the same mobility as inorganic phosphorothioate and which migrated ahead of the ultraviolet-absorbing component. Reprecipitation of the barium salt did not remove the inorganic phosphorothioate. To obtain the nucleotide as its more water-soluble potassium salt, an aqueous solution of the barium salt (150 mg) was passed through a column of Dowex 50 (K^+ form) and the effluent and washings were lyophilized to dryness. A solution of the residue in water (0.5 ml) was applied to two sheets (18 × 22 in.) of Whatman No. 3MM paper (previously washed with ammonium formate by ascending chromatography) and subjected to electrophoresis for 1 hr at 30 V/cm in 0.05 M ammonium formate buffer (pH 6.2). The single ultraviolet-absorbing band which was observed was eluted with water. The solution was concentrated by lyophilization and the product was precipitated at pH 8 as its barium salt by addition, in succession, of barium acetate, $Ba(OH)_2$, and ethanol. This material moved as a single spot on chromatograms in solvents A, B, C and D (R_F values 0.09, 0.58, 0.76, and 0.30, respectively. The values for IMP were 0.09, 0.48, 0.80, and 0.30). The electrophoretic mobilities at pH 3.5 and 8 were the same as those of IMP. In the borate buffer of pH 9.2 the mobility was 17.5 cm/hr (IMP, 16.5 cm/hr). At pH 5 the product had λ_{max} 248 mμ, λ_{min} 223 mμ; at pH 12 λ_{max} was 253 mμ, λ_{min} 226 mμ. Potentiometric titration gave a pK_a value of 5.2 for the secondary phosphate ionization. The corresponding value for IMP was found to be 6.3. The secondary dissociation of phosphorothioic acid is 5.75 (Neumann *et al.*, 1965).

Anal. Calcd for $C_{10}H_{11}BaN_4O_7PS$: C, 24.0; H, 2.2; N, 11.2; P, 6.2; S, 6.4. Found: C, 23.9; H, 2.8; N, 10.9; P, 6.1; S, 6.3.

When the reaction was carried out in acetonitrile which had been dried over P_2O_5 the yield was reduced. Substitution of *N*,*N*-dimethylformamide or tetrahydrofuran for acetonitrile gave no product.

Phosphorylation of 2',3'-O-Isopropylidene Inosine with Triimidazolyl 1-Phosphinsulfide. 2',3'-*O*-Isopropylideneinosine (0.1 g) in 2 ml of dry pyridine was treated with triimidazolyl 1-phosphinsulfide (0.17 g, 2 equiv) and 3 drops of triethylamine at room temperature for 18 hr. Eckstein (1966) employed no triethylamine and a reaction time of 12 hr for phosphorylation of 2',3'-*O*-isopropylideneuridine with this reagent. Paper chromatography of the mixture revealed much unchanged starting material. Volatiles were removed under reduced pressure and a solution (pH 2) of the residue in aqueous acetic acid was kept at 70–80° for 1 hr. P_i was precipitated with $Ba(OH)_2$ at pH 8 and two volumes of ethanol was added. The resulting precipitate showed a higher R_F in system A and a lower R_F in system B than IMP and inosine 5'-*O*-phosphorothioate; its electrophoretic mobility in phosphate buffer of pH 8 and formate buffer of pH 3.5 was the same as for IMP

[1] Abbreviations used are: 5'-thio-IMP, 5'-mercapto-5'-deoxyinosine 5'-*S*-phosphate; 5'-amino-IMP, 5'-amino-5'-deoxyinosine 5'-*N*-phosphate.

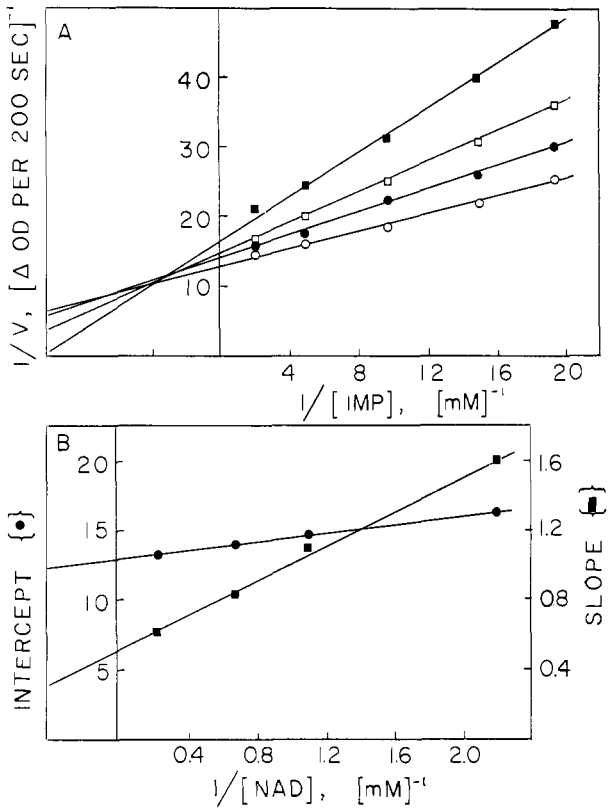


FIGURE 2: Initial velocity data for IMP dehydrogenase at pH 6.0. Frame A: IMP varied at fixed NAD concentrations of 4.6×10^{-4} M (■), 9.2×10^{-4} M (□), 1.5×10^{-3} M (●), and 4.6×10^{-3} M (○). Frame B: replots of the slopes and intercepts *vs.* the reciprocal of the NAD concentration. Optical density changes measured at 340 mμ.

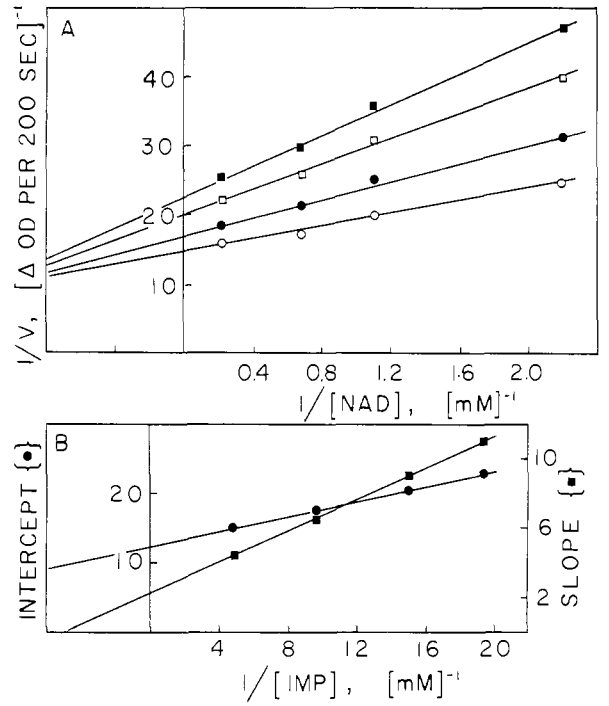


FIGURE 3: Initial velocity data for IMP dehydrogenase at pH 6.0. Frame A: NAD varied at fixed IMP concentrations of 5.2×10^{-5} M (■), 6.7×10^{-5} M (□), 1.0×10^{-4} M (●), and 2.1×10^{-4} M (○). Frame B: replots of the slopes and intercepts *vs.* the reciprocal of the IMP concentration.

and inosine 5′-*O*-phosphorothioate, but in borate buffer of pH 9.2 its mobility was significantly less than that of these two nucleotides, presumably because of inability to form the negatively charged diol–borate complex. On chromatograms the product reacted negatively to the periodate spray and positively to the spray for phosphorus. When the time for acidic hydrolysis of the reaction mixture was doubled, no nucleotide was isolated. The triimidazolyl 1-phosphinsulfide used in these experiments was prepared by the procedure of Eckstein (1966) and was identical with a sample kindly provided by Dr. Eckstein.

5′-Mercapto-5′-deoxyinosine 5′-S-Phosphate. A suspension of 0.3 g of 5′-iodo-5′-deoxyinosine (Hampton *et al.*, 1968) and 1.5 g of trisodium phosphorothioate in 30 ml of water was stirred at 22° for 36 hr. Phosphorylation was almost complete as indicated by electrophoresis in ammonium formate buffer (pH 6.2). Most of the excess of trisodium phosphorothioate was removed by filtration after addition of 60 ml of water and 180 ml of methanol. After precipitation of additional inorganic thiophosphate at pH 8 with aqueous barium acetate the product was isolated as a barium salt (0.2 g, 59% yield) by addition of two volumes of ethanol. The product gave a single ultraviolet-absorbing spot on paper chromatography and electrophoresis. The R_F values (0.09 and 0.80) in solvents A and C, respectively, were the same as those of IMP; in solvent E the R_F was 0.60 (IMP, 0.65). The electrophoretic mobility at pH 8 and 9.2 (15.5 and 16.5 cm per hr, respectively) was also the same as for IMP; spectral data: λ_{max} 248 mμ at pH 5, 253 mμ at pH 12; λ_{min} 223 mμ at pH 5, 224 mμ at pH 12. The pK_a value for secondary phosphate ionization was 5.7. For comparison, the corresponding value for *S-n*-butylphosphorothioic acid is 5.5 (Dittmer *et al.*, 1963).

Anal. Calcd for $C_{20}H_{24}BaN_8O_{14}P_2S_2$: C, 27.8; H, 2.8; N, 12.9. Found: C, 28.0; H, 3.4; N, 13.3.

Assay with IMP dehydrogenase of the amount of NADH produced from a spectrophotometrically determined amount of the compound indicated a purity of 95–98% on the assumption that at pH 5 the compound has the same extinction coefficient as IMP at 248 mμ. A solution of the product in aqueous 2.5% acetic acid was boiled under reflux for 20 min. Paper chromatography in solvent A showed a single ultraviolet-absorbing spot with the same R_F (0.23; inosine and hypoxanthine both have R_F 0.51) as 5′-mercapto-5′-deoxyinosine (Hampton *et al.*, 1968). To an aqueous suspension of the barium 5′-deoxy-5′-mercapto-IMP was added aqueous KI-I_2 until the iodine color persisted. A sample of 5′-mercapto-5′-deoxyinosine was oxidized in the same way. Paper chromatography in system A revealed that in both cases complete oxidation to a single ultraviolet-absorbing product (R_F 0.17) had occurred.

5′-Amino-5′-deoxyinosine 5′-N-Phosphate. 5′-Amino-5′-deoxyinosine (0.3 g; Hampton *et al.*, 1968) was added to a mixture of 1 ml of acetonitrile (Fisher Certified), 0.1 ml of dry pyridine, and 0.42 ml of $POCl_3$ at 0–5°. The mixture was stirred for 2 days at 0–5° and added to a suspension of 1 g of $Ba(OH)_2$ in 25 ml of saturated aqueous $Ba(OH)_2$ and 25 g of ice and the pH was kept at 8 by addition of $Ba(OH)_2$. After a further 0.5 hr barium phosphate was centrifuged and barium nucleotides (0.15 g) precipitated by addition of two volumes of

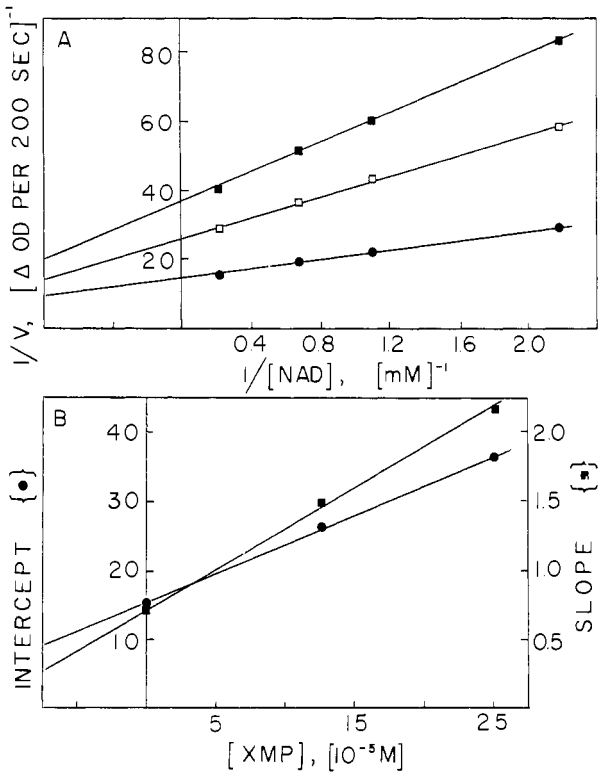


FIGURE 4: Frame A: reciprocal plot with NAD as the variable substrate and XMP as product inhibitor of IMP dehydrogenase at pH 6.0. IMP was 1.0×10^{-4} M. The XMP concentrations were 0 (●), 1.25×10^{-4} M (□), and 2.5×10^{-4} M (■). Frame B: secondary plots of slopes and intercepts against the XMP concentration.

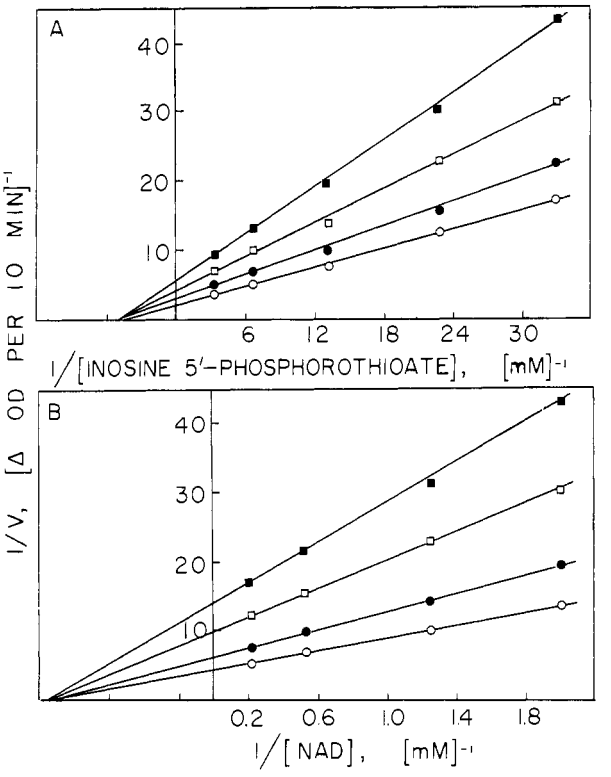


FIGURE 5: Initial velocity data for inosine 5′-phosphorothioate and IMP dehydrogenase at pH 8.1. Frame A: inosine 5′-phosphorothioate varied at fixed NAD concentrations of 4.7×10^{-4} M (■), 7.9×10^{-4} M (□), 1.9×10^{-3} M (●), and 4.7×10^{-3} M (○). Frame B: NAD varied at fixed inosine 5′-phosphorothioate concentrations of 3.0×10^{-5} M (■), 4.5×10^{-5} M (□), 7.5×10^{-5} M (●), and 3.0×10^{-4} M (○). Optical density changes measured at 290 mμ.

ethanol. This product was converted into the potassium salt with Dowex 50 (K^+) ion-exchange resin and subjected to paper electrophoresis in ammonium formate buffer (pH 8) by the procedure described for purification of inosine 5′-*O*-phosphorothioate. The principal ultraviolet-absorbing band (mobility 10.3 cm/hr) corresponded to 5′-amino-5′-deoxyinosine 5′-*N*-phosphate; a second ultraviolet-absorbing band (mobility 18 cm/hr) was also present. The purified product was obtained as a barium salt as described for inosine 5′-phosphorothioate. The solid was initially white and became yellow after several days at room temperature; discoloration was slower at lower temperatures. The product was homogeneous upon electrophoresis at pH 9.2 (mobility 15.5 cm/hr; IMP was 16.5 cm/hr) and upon paper chromatography in systems A, C, and E in which it had the same R_F values (0.09, 0.80, and 0.65, respectively) as IMP. At pH 5 it had λ_{max} 249 mμ, λ_{min} 223 mμ, and at pH 12 it had λ_{max} 252 mμ, λ_{min} 223 mμ. The pK_a value for ionization at the phosphoramidate nitrogen was 8.5. The reported values for phosphoramidic acid are 3.0 and 8.2 (Chanley and Feageson, 1963).

TABLE I: Kinetic Parameters for Substrates of IMP Dehydrogenase.

Analog	pH	K_{IMP}[a] (M × 10^5)	K_{NAD}[b] (M × 10^3)	$K_{i\ IMP}$[c] (M × 10^5)	Rel V_{max}
IMP	8.1	2.0	1.0	2.0	1.00
	6.0	4.2	0.2	19.5	0.03
Inosine 5′-phosphorothioate	8.1	21.0	0.9	23.0	1.05
5′-Thio-IMP	8.1	1.3	5.3	1.7 1.9[d]	0.75
5′-Amino-IMP	8.1	3.8	2.0	0.5	0.67
	6.0	7.4	0.4	13.0	0.01

[a] Michaelis constant for IMP and its analogs. [b] Michaelis constant for NAD. [c] Dissociation constant for IMP and its analogs. [d] Determined by inhibition experiments with IMP.

Hydrolysis of the product at 22° was followed by electrophoresis at pH 8 which readily separated the cationic 5′-amino-5′-deoxyinosine from the anionic inosine 5′-*N*-phosphate; hydrolysis was as follows: pH 1, 0.5 hr, 100%; pH 3, 0.5 hr, 33%; pH 5 (sodium acetate buffer), 1 hr, 45%; and pH 10 (glycine buffer), 1 hr, 0–5%. Ultraviolet-absorbing products of hydrolysis other than 5′-amino-5′-deoxyinosine were not detected. The half-life of phosphoramidic acid in aqueous solution at 20° and pH 5 is calculated from the first-order rate constant of Chanley and Feageson (1963) to be 27 hr. The present hydrolysis rates as a function of pH tend to resemble the S-shaped pH *vs.* rate profile found for phosphoramidic acid by the same workers. That 5′-amino-IMP

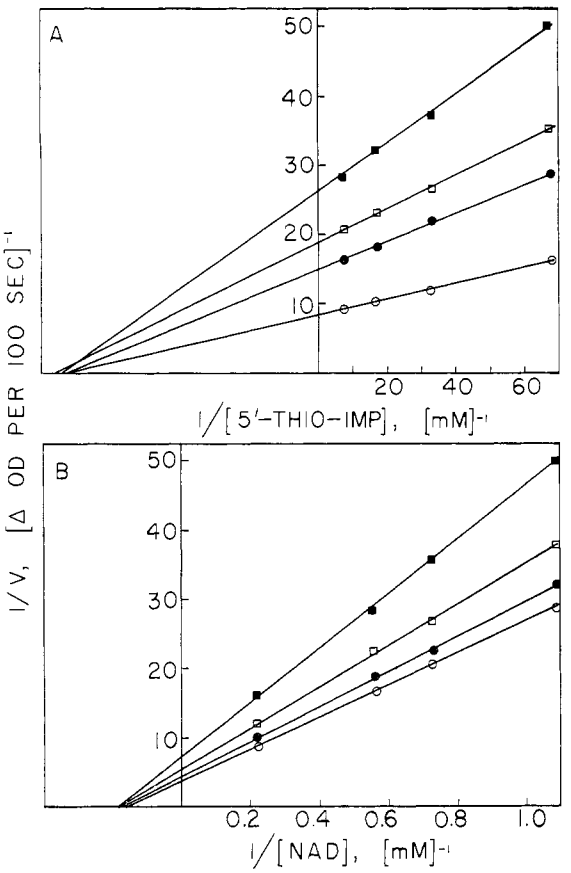


FIGURE 6: Initial velocity data for 5'-thio-IMP and IMP dehydrogenase at pH 8.1. Frame A: 5'-thio-IMP varied at fixed NAD concentrations of 9.2×10^{-4} M (■), 1.4×10^{-3} M (□), 1.8×10^{-3} M (●), and 4.6×10^{-3} M (○). Frame B: NAD varied at fixed 5'-thio-IMP concentrations of 1.5×10^{-5} M (■), 3.0×10^{-5} M (□), 6.0×10^{-5} M (●), and 1.2×10^{-4} M (○). Optical density changes measured at 290 mμ.

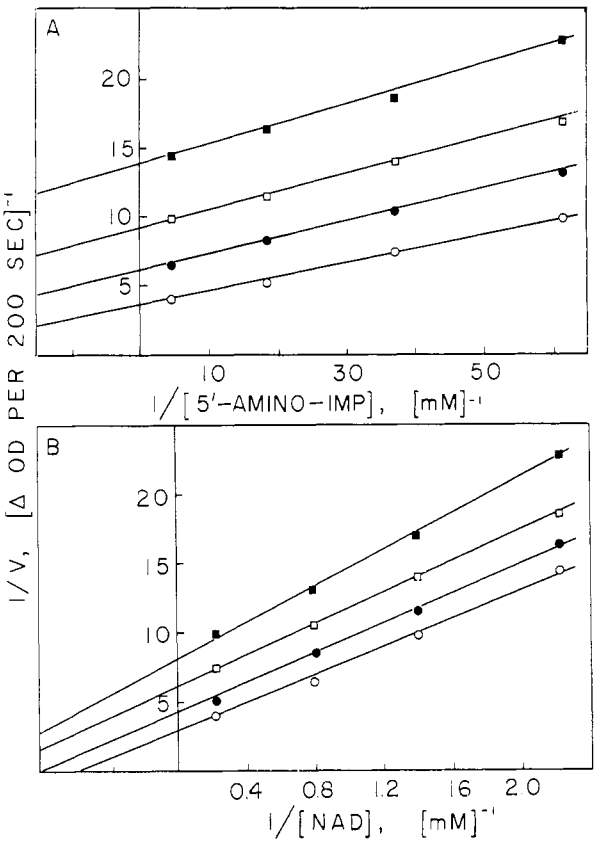


FIGURE 7: Initial velocity data for 5'-amino-IMP and IMP dehydrogenase at pH 8.1. Frame A: 5'-amino-IMP varied at fixed NAD concentrations of 4.5×10^{-4} M (■), 7.2×10^{-4} M (□), 1.3×10^{-3} M (●), and 4.5×10^{-3} M (○). Frame B: NAD varied at fixed 5'-amino-IMP concentrations of 1.6×10^{-5} M (■), 2.7×10^{-5} M (□), 5.0×10^{-5} M (●), and 2.2×10^{-4} M (○). Optical density changes measured at 340 mμ.

hydrolyzes faster than phosphoramidic acid itself is presumably due to the base-strengthening effect of the alkyl residue attached to the phosphoramidate nitrogen. The same effect serves to account for the more rapid hydrolysis at pH 4 and 5 of a phosphoromorpholidate than the corresponding phosphoramidate (Moffatt and Khorana, 1961).

An aqueous solution (5×10^{-5} M) of the barium salt of 5'-amino-IMP was titrated with sodium periodate by the spectrophotometric method of Dixon and Lipkin (1954); the uptake of periodate was 90–95% that by sodium IMP in a parallel experiment.

Kinetics at pH 6.0 of IMP Dehydrogenase. Figures 2 and 3 show the initial velocity data, plotted in reciprocal form, for IMP dehydrogenase at pH 6.0. An intersecting pattern of lines is obtained for which slopes and intercepts are linear functions of the reciprocal of the fixed substrate concentrations. The point of intersection for these reciprocal plots is in the upper left-hand quadrant and not on the horizontal $1/S$ axis as was the case at pH 8.1 (Brox and Hampton, 1968). Figure 4 shows that XMP is a linear noncompetitive inhibitor of NAD. This was also the case at pH 8.1. The dissociation constant, K_{iq}, of XMP was 6.4×10^{-5} M. At pH 8.1 the value of K_{iq} was 12×10^{-5} M (Brox and Hampton, 1968). Since a random kinetic mechanism should give rise to competitive inhibition (Cleland, 1963), it is concluded that the ordered BiBi model applicable at pH 8.1 may still be used at pH 6.0. The numerical values for kinetic parameters of IMP dehydrogenase at pH 6.0 together with their equivalent values at pH 8.1 are given in Table I.

Kinetic Studies with the IMP Analogs. The IMP analogs were all substrates of IMP dehydrogenase. The initial velocity data for these compounds at pH 8.1 are shown in Figures 5–7. Replots of all slopes and intercepts were linear. No product inhibition experiments were undertaken so the kinetic parameters listed in Table I were calculated utilizing rate equations for the ordered BiBi kinetic model presented previously (Brox and Hampton, 1968). The rate equations were derived by the method of King and Altman (1956) and expressed in the terminology of Cleland (1963).

The reduced maximal velocity and higher Michaelis constant for NAD with 5'-thio-IMP resulted in little of this analog being oxidized under the normal assay conditions employed with IMP as the substrate. Hence, it was possible to use this analog as an inhibitor of the enzyme-catalyzed oxidation of IMP. Figure 8 shows that 5'-thio-IMP gave linear competitive inhibition as the IMP concentration was varied. The dissociation constant of 1.9×10^{-5} M for this analog as determined from the horizontal intercept of the replot is in good agreement with the value of 1.7×10^{-5} M obtained from

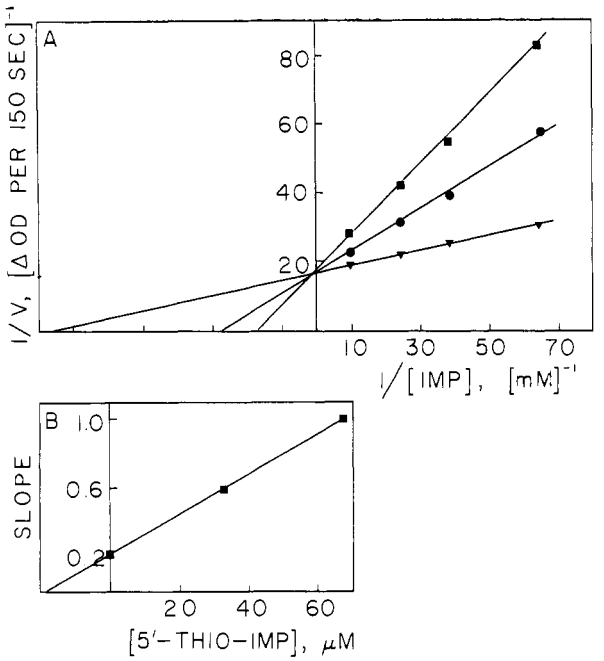


FIGURE 8: Inhibition of IMP dehydrogenase by 5′-thio-IMP. Frame A: IMP varied with 5′-thio-IMP concentrations of 0 (▼), 3.3 × 10^{-5} M (●), and 6.7 × 10^{-5} M (■). The NAD concentration was fixed at 1 × 10^{-3} M. Frame B: replot of slopes *vs.* 5′-thio-IMP concentration.

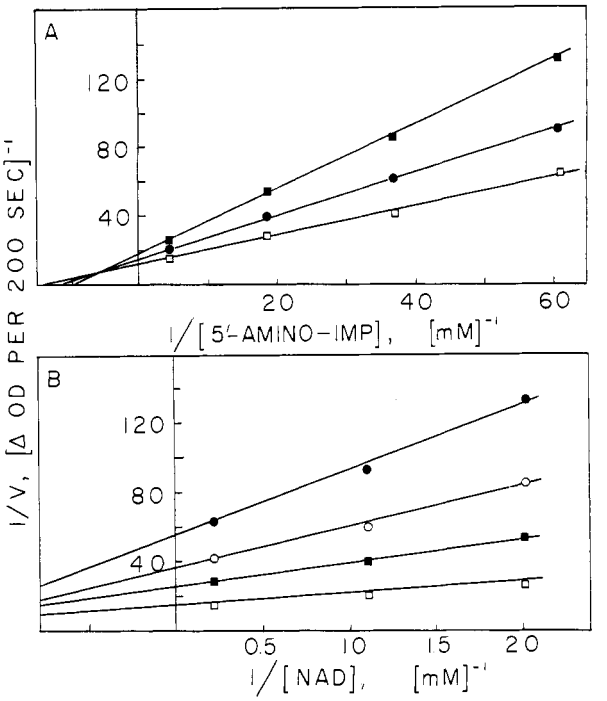


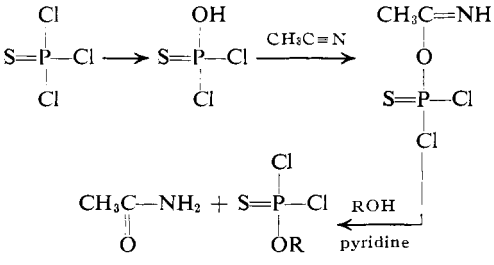
FIGURE 9: Initial velocity data for 5′-amino-IMP and IMP dehydrogenase at pH 6.0. Frame A: 5′-amino-IMP varied at fixed NAD concentrations of 4.5 × 10^{-4} M (■), 1.1 × 10^{-3} M (●), and 4.5 × 10^{-3} M (□). Frame B: NAD varied at fixed 5′-amino-IMP concentrations of 1.6 × 10^{-5} M (●), 2.7 × 10^{-5} M (○), 5.5 × 10^{-5} M (■), and 2.2 × 10^{-4} M (□). Optical density changes measured at 340 mμ.

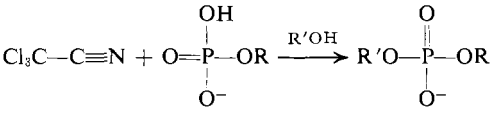
initial velocity data (Table I). This consistency is additional evidence that the ordered BiBi kinetic model is a satisfactory working model for the enzyme. The initial velocity data for 5′-amino-IMP at pH 6.0 are shown in Figure 9; for comparison with results at pH 8.1, see Figure 7.

As with IMP, the equilibrium for oxidation of these analogs by IMP dehydrogenase is very much to the right, since with excess of NAD the final optical density change at 340 mμ was greater than 95% of the theoretical as calculated spectrophotometrically using the extinction coefficient of IMP for all the IMP analogs.

Discussion

Preparation of inosine 5′-phosphorothioate was attempted initially by treatment of 2′,3′-*O*-isopropylideneinosine with triimidazolyl phosphinsulfide. Although this procedure has yielded several pyrimidine nucleoside 5′-phosphorothioates (Eckstein, 1966), phosphorylation in the present case required strong base catalysis and attempts to remove the imidazole and isopropylidene blocking groups from the product resulted in extensive decomposition. Treatment of 2′,3′-*O*-isopropylideneinosine with thiophosphoryl chloride in acetonitrile in the presence of pyridine resulted in extensive phosphorylation provided that water was not rigorously excluded. Substitution of acetonitrile by *N*,*N*-dimethylformamide considerably reduced the extent of phosphorylation. Promotion of the phosphorylation by acetonitrile and water suggests the involvement of an acetimidoyl phosphate intermediate formed from condensation of thiophosphorodichloridic acid with acetonitrile. Analogous intermediates have been proposed to result from interaction of phosphomonoesters with trichloroacetonitrile (Cramer and Weimann, 1961) and with acetonitrile (Clark *et al.*, 1966) and to be readily subject to nucleophilic attack on phosphorus.





5′-Thio-IMP was conveniently prepared by condensation of 5′-iodo-5′-deoxyinosine (Hampton *et al.*, 1968) with trisodium phosphorothioate. That the product was an *S*-alkyl phosphorothioate was confirmed by its facile P–S cleavage under acidic conditions (Wieland and Lambert, 1956) to give the known 5′-mercapto-5′-deoxyinosine (Hampton *et al.*, 1968) and its oxidation by iodine (Wieland and Lambert, 1956) to a compound chromatographically identical with the disulfide obtained by similar oxidation of 5′-mercapto-5′-deoxyinosine. The third analog, 5′-amino-IMP, was obtained as the major product upon treatment of 5′-amino-5′-deoxyinosine with phosphorus oxychloride in acetonitrile and its structure

TABLE II: Kinetic Parameters as Functions of Rate Constants.[a]

Kinetic Parameter	Expression
$V_{forward}$	$\frac{k_5k_7[E_t]}{(k_5 + k_7)}$
K_{ia}	$\frac{k_2}{k_1}$
K_a	$\frac{k_5k_7}{k_1(k_5 + k_7)} = \frac{V_f}{k_1[E_t]}$
K_b	$\frac{k_7(k_4 + k_5)}{k_3(k_5 + k_7)}$

[a] $V_{forward}$ is the maximum velocity in the forward direction, K_{ia} is the dissociation constant of IMP, K_a is the Michaelis constant of IMP, and K_b is the Michaelis constant of NAD.

was established by its electrophoretic mobility, its rate of acid-catalyzed P–N bond cleavage, and its uptake of 1 molar equivalent of periodate indicative of lack of substitution at the 2′,3′-diol system. Phosphorylation with phosphorus oxychloride in triethyl phosphate, which is reported to occur exclusively at the 5′ position of unprotected ribonucleosides (Yoshikawa *et al.*, 1967) did not produce higher yields of 5′-amino-IMP.

The phosphate moiety of IMP presumably assists the specific binding of IMP directly, *i.e.*, by interacting with groups on the enzyme, but, in addition, the phosphate might assist by conferring on the IMP the particular conformation most favorable for binding. IMP and other purine ribonucleoside 5′-phosphates almost certainly exist in aqueous solution predominantly in the *anti* conformation in the light of recent proton magnetic resonance data (Schweizer *et al.*, 1968; Danyluk and Hruska, 1968). Purine ribonucleosides, on the other hand, have optical rotatory dispersion characteristics (Cotton effects) which seem to suggest that they exist mainly in the *syn* conformation in aqueous solution, although this question is presently far from resolved (Klee and Mudd, 1967; Miles *et al.*, 1967; Ikehara *et al.*, 1967; Hampton and Nichol, 1967). Nevertheless, these findings suggested that the lack of affinity of inosine, at high concentration, for the IMP site of IMP dehydrogenase (Nichol *et al.*, 1967) might be partly due to conformational differences between inosine and IMP. A major difficulty in assigning to the nucleosides conformations markedly different from those of the nucleotides is the close similarity between their respective optical rotatory dispersion curves. This has been reported for adenosine and AMP and guanosine and GMP (Yang *et al.*, 1966), and Figure 10 shows that this similarity extends to inosine and the dianion of IMP (phosphate, $pK_a = 6.3$) and is independent of enolic ionization ($pK_a = 8.7$) at the purine 6-oxygen. This ionization is associated with a shift in absorption maximum from 248 to 253 mμ, and the observed displacements in the optical rotatory dispersion profiles appear to reflect this spectral shift rather than a change in the spatial relationship of the chromophoric purine ring to the furanose ring. Figure 10 also shows the

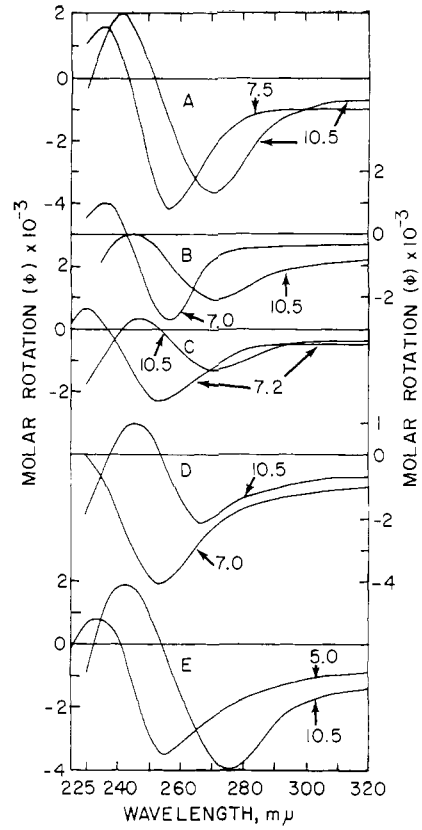
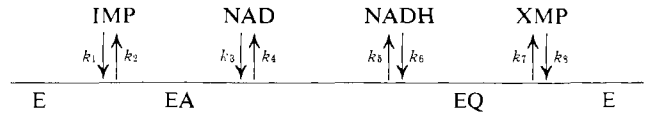


FIGURE 10: Ultraviolet rotatory dispersion curves in aqueous buffers. (A) IMP, (B) inosine 5′-phosphorothioate, (C) 5′-thio-IMP, (D), 5′-amino-IMP, and (E) inosine. The numerals indicate pH values of the buffers.

optical rotatory dispersion characteristics of the IMP analogs of the present study at pH values at which the phosphate is principally in the dianion form. All the analogs showed negative Cotton effects and had profiles similar to those of IMP except for small variations in the magnitude of rotations which do not correlate with the enzyme–substrate dissociation constants of Table I. Optical rotatory dispersion measurements at lower pH values were precluded by the acid lability of 5′-thio-IMP and 5′-amino-IMP.

Initial velocity and product inhibition studies of IMP dehydrogenase of *A. aerogenes* (Brox and Hampton, 1968) led to an ordered BiBi kinetic model, as shown. Table II shows for this model some kinetic parameters expressed in terms of



the individual first- and second-order rate constants. The initial velocity data for the present series of IMP analogs were consistent with this ordered model, and the kinetic parameters of Table I were calculated utilizing the ordered BiBi equations previously used for IMP itself.

When inosine 5′-phosphorothioate replaces IMP as a substrate, there is no significant change in V_{max} or Michaelis constant for NAD (Table I). The expressions of Table II show that no marked changes have occurred in the rates of release

of products which are described by the k_5 and k_7 rate constants. Since K_a is equal to V_{max}/k_1E_t, it follows that k_1 must have decreased by a factor of ten to give rise to the observed tenfold increase in Michaelis constant coupled with the unchanged V_{max}. Furthermore, since a tenfold increase in K_{ia} is observed, it follows that k_2 is unchanged. The results with this analog may hence be explained by a decrease in a single rate constant, namely, that which describes the rate at which the analog and enzyme interact. Most simply, this could be a steric effect due to substitution of the larger sulfur for one of the phosphate oxygens. Chemical evidence indicates that the dianion of phosphorothioic acid exists mainly in the $(HO)POS^{-}O^{-}$ form (Pollack and Friedman, 1966); binding of the present IMP analog could, therefore, be influenced by localization of negative charge on sulfur at the expense of the two oxygens in the anionic species.

In the case of 5′-thio-IMP the 25% reduction in V_{max} appears to account for the slight decrease in Michaelis constant and shows that k_1 must be the same as with IMP. Since the dissociation constant, K_{ia}, is the same as for IMP, it follows that k_2 also must be unaltered. This indicates that the C-5′ oxygen of IMP does not significantly contribute to total binding energy. This oxygen could act as an electron donor in hydrogen-bond formation with a group on the enzyme, but this possibility seems unlikely because 5′-thio-IMP binds to the enzyme as well as IMP, yet the electron pair of sulfur enters into such hydrogen bonding much less readily than in the case of oxygen. At the pH (8.1) at which the enzyme–substrate dissociation constants were determined, both IMP (pK_a = 6.3) and 5′-thio-IMP (pK_a = 5.7) are present predominantly (98.4 and 99.6%, respectively) as dianionic phosphomonoesters, and the dissociation constants thus refer to those ionic species. Were IMP and 5′-thio-IMP bound exclusively in their monoanion form, the kinetic data indicate that 5′-thio-IMP would bind more strongly than IMP, and the conclusion regarding noninvolvement in binding of the 5′-oxygen of IMP would remain valid.

Substitution of 5′-amino-IMP for IMP resulted in a decreased maximal velocity coupled with a twofold increase in Michaelis constant for which a threefold reduction in the k_1 rate constant is required. Since the observed dissociation constant is four times less than that of IMP, it follows that k_2 for 5′-amino-IMP is twelve times smaller than k_2 for IMP. It seems probable that the monoanion form ($HNPO_3H^{-}$) of 5′-amino-IMP, like that of unsubstituted phosphoramidic acid, exists in aqueous solution largely as the zwitterion, $NH_2^{+}PO_3^{2-}$. X-Ray diffraction has revealed a zwitterionic structure for crystalline monosodium phosphoramidate (Hobbs *et al.*, 1953) and comparison of pK_a values of phosphoramidic acid and related compounds together with rate studies of acidic hydrolysis of phosphoramidates (Chanley and Feageson, 1963) is strongly suggestive of a zwitterion structure for the phosphoramidate monoanion in solution. The 5′-amino-IMP (pK_a = 8.5) is hence probably present at the pH of the above studies as a mixture of $NH_2^{+}PO_3^{2-}$ (70%) and $NHPO_3^{2-}$ (30%) and kinetic studies were therefore conducted at pH 6 in order to evaluate solely the zwitterionic species. Product inhibition and initial velocity studies at pH 6 indicated no deviation of the kinetic mechanism from the ordered BiBi model at pH 8.1, and the enzyme–substrate dissociation constants at pH 6 of IMP and 5′-amino-IMP were therefore determined from initial velocity data in the same manner as were the dissociation constants at the higher pH value. The results (Table I) show that at pH 6 the k_1 rate constant of 5′-amino-IMP is one-sixth the k_1 of IMP and the k_2 constant is one-tenth the k_2 of IMP; these relationships are not markedly different from those at pH 8.1. The reduced k_1 for 5′-amino-IMP could result from electrostatic repulsion between positive centers at the enzymic phosphate binding site and the protonated amide nitrogen of this analog. Combination of the 5′-amino-IMP zwitterion with the above positive centers would be expected to markedly reduce electron availability at the phosphoramide nitrogen and result in simultaneous release of its proton. In this way, the factor (electrostatic repulsion) which operated to reduce k_1 would not tend to increase k_2. The observed decrease in k_2 might originate from the acid-weakening effect of the deprotonated phosphoramide nitrogen which would tend to favor a less readily dissociable salt linkage between the phosphate moiety and basic centers at its binding site.

The values of the kinetic parameters of IMP at pH 6 (Table I) show that the reduction in pH results in a 70-fold reduction in k_1 and a 6-fold reduction in k_2. In the case of 5′-amino-IMP, the reductions in k_1 and k_2 associated with the pH change are of similar magnitude, namely, 132- and 5-fold, respectively. If substrate binding involves only the dianionic species, as seems most probable (see below), then at pH 6–8 relatively little change occurs in the proportion of these species, and the large changes in k_1 and k_2 shared by both substrates probably reflect modification of the IMP binding site and/or of the rate of the conformational change which may occur in this enzyme prior to specific binding of NAD (Nichol *et al.*, 1967).

At pH 8.1, 5′-amino-IMP (primary phosphate pK_a = *ca.* 3.3), if present exclusively as zwitterions, would furnish 1000 times less phosphoryl "monoanion" ($NH_2^{+}PO_3H^{-}$) than IMP (secondary pK_a = 6.3). At the same pH the enzyme–substrate dissociation constant of 5′-amino-IMP is one-quarter that of IMP. This would indicate that these phosphomonoesters are not binding in the monoanion form but rather in the dianion form. However, it is possible that binding of monoanionic 5′-amino-IMP could be strengthened by a hydrogen bond to the enzyme from a hydrogen of its PNH system, and were the electron donor group on the enzyme favorably situated for strong bond formation, the energy release could be high enough (4.2 kcal/mole) to decrease the dissociation constant by a factor of 1000 to produce the observed value. This eventuality, however, appears improbable. The findings with 5′-thio-IMP also tend to favor the conclusion that the dianion form is preferentially bound, since this analog binds as well or better than IMP yet furnishes only one-fourth as much monoanion form.

Acknowledgments

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