Studies on Phosphate Binding Sites of Inosinic Acid Dehydrogenase and Adenylosuccinate Synthetase*

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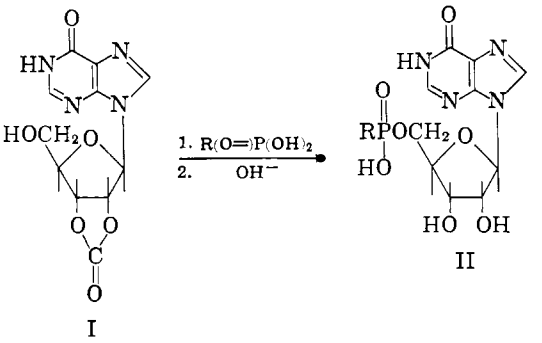
ABSTRACT: Inosine 5′-methylphosphonate, inosine 5′-phosphorofluoridate, and inosine 5′-phosphite have been prepared and tested, together with related compounds, as inhibitors of inosine 5′-phosphate (IMP) dehydrogenase of *Aerobacter aerogenes* and adenylosuccinate synthetase of *Escherichia coli*. With both enzymes, inosine 5′-phosphorofluoridate and 5′-chloromethylphosphonate did not act as reagents for the phosphate binding sites of IMP. Inorganic phosphate was a feeble inhibitor and gave noncompetitive kinetics with the synthetase and mixed kinetics with the dehydrogenase. Ribose 5-phosphate (4 mM) had little effect on the synthetase and inhibited IMP dehydrogenase noncompetitively; at a level of 10 mM it inhibited IMP dehydrogenase in partly competitive fashion. Inosine and the above IMP analogs were not substrates and were weak noncompetitive inhibitors of both enzymes. Binding of IMP may require a conformational change in the enzyme mediated by the phosphomonoester portion of IMP. Properties of the fluorine of inosine 5′-phosphorofluoridate show that the phosphomonoester binding is unlikely to involve an enzyme–phosphorus bond. A hydrogen bond between a phosphate oxygen and a hydrogen of the enzymes also appears unlikely. If the phosphate moiety of IMP binds as the monoanion, the process might include hydrogen bonding through the ionizable hydroxyl and also an electrostatic or covalent linkage from a second phosphate oxygen. Inosine 5′-phosphorofluoridate and 5′-chloromethylphosphonate did not markedly inhibit or inactivate spleen or venom phosphodiesterase, pancreatic ribonuclease, or adenosine monophosphate deaminase. The phosphorofluoridate was a substrate of venom diesterase.

Phosphomonoester substrates can bind to their specific enzymic sites by a large number of possible modes encompassing electrostatic and/or hydrogen bonds (discussed by Baker *et al.*, 1965) and conceivably also bonds with appreciable covalent character; these latter could be between the enzyme and either the oxygen or the phosphorus of the phosphate group. Studies of the pH dependence of substrate binding and of phosphate association constants carried out with fructose 1,6-diphosphate aldolase have indicated the binding in that case is electrostatic and involves the action of cooperating positive charges located at the phosphate binding site (Mehler, 1963; Ginsberg and Mehler, 1966). The present report describes studies of the binding of the phosphomonoester portion of inosine 5′-phosphate[1] (II, R = OH) to adenylosuccinate synthetase[2] and to IMP dehydrogenase.[3] Inosine 2′,3′-carbonate (I) (Scheme I) has been converted to analogs of IMP (II) in which one hydroxyl of the phosphate moiety is replaced by another group. Inosine 5′-phosphite and inosine 5′-methylphosphonate (II, R = H and CH_3) were prepared to help delineate structural requirements for binding to the IMP sites, whereas inosine 5′-phosphorofluoridate and inosine 5′-chloromethylphosphonate (II, R = F and CH_2Cl) were of interest as potential selective phosphorylating and alkylating agents, respectively, for

SCHEME I



I II

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[1] Abbreviations used: IMP, inosine 5′-phosphate; NAD, nicotinamide–adenine dinucleotide; AMP, adenosine monophosphate; GTP, guanosine triphosphate; TMS, tetramethylsilane.

[2] IMP:L-aspartate ligase (GDP), EC 6.3.4.4.

[3] IMP:NAD oxidoreductase, EC 1.2.1.14.

the phosphate binding sites of IMP. Kinetic data is presented for inhibition of the two enzymes by these IMP analogs and by inosine, ribose 5-phosphate, and inorganic phosphate. The results suggest that IMP may help to induce formation of its binding site on the enzymes. Both of the ionizable hydroxyls of the phosphate moiety of IMP appear to be necessary for specific binding. Two of the IMP analogs (II, R = F and CH_2Cl) have been tested as inhibitors of AMP deaminase, pancreatic ribonuclease, and two phosphodiesterases.

Recent evidence (summarized by Brockman and Chumley, 1965) shows that various antineoplastic purine analogs, in the form of their ribonucleoside 5′-phosphates, inhibit enzymes at several sites on the pathways of purine ribonucleotide biosynthesis and interconversions. Inasmuch as the substrates of all enzymes of these pathways are phosphomonoesters, the present studies of phosphomonoester binding were carried out to assist the design of new inhibitors of purine nucleotide biosynthesis and hence, conceivably, of new antineoplastic drugs.

Materials and Methods

Chemicals. Chemicals for the assay of inosinic acid dehydrogenase were obtained from the sources listed previously (Hampton, 1963). For the assay of adenylosuccinate synthetase L-aspartic acid and sodium GTP were the highest quality available from Sigma Chemical Co.[4] Anions other than IMP and GTP were used as the potassium salts and these were obtained by passage through the potassium form of Dowex 50 ion-exchange resin. Inosine 5′-chloromethylphosphonate was prepared by the method of Hampton and Nichol (1966).

Enzyme Preparations and Assays. IMP dehydrogenase of *Aerobacter aerogenes* was purified to the step 4 stage and assayed by method b of Hampton and Nomura (1967) but with 0.1 mM IMP. The same enzyme preparation was used throughout; 0.01 ml of this fraction caused an increase in optical density at 340 mμ of 0.018/min. AMP deaminase (1–2 μM units/ml) was from Sigma Chemical Co. and diesterases from Worthington Biochemical Corp.

Adenylosuccinate synthetase was purified from *Escherichia coli* strain B (supplied by the Grain Processing Corp., Muscatine, Iowa) by the method of Lieberman (1956) to the low pH, fraction II stage. These purification steps were completed within a period of a few hours since considerable loss of activity occurred when the less purified fractions were stored overnight at 2°. Assays were carried out by following the increase in optical density at 280 mμ due to the conversion of IMP to adenylosuccinate. In a 1-cm light-path cuvet was added a stock solution (0.4 ml) consisting of 0.5 M glycine buffer, pH 8.0 (6 ml), 0.01 M L-aspartic acid (1 ml), and 0.1 M magnesium chloride (1 ml). IMP (0.005 M) was then added to give the desired final concentration followed by the inhibitor solution, if any, and the enzyme preparation (0.1 ml). Water was added to bring the volume to 0.9 ml. The mixture was placed in the spectrophotometer and after 5 min the reaction was started by the addition of 0.001 M GTP (0.1 ml). The resulting changes in optical density were recorded for at least 10 min at 23–25° against a blank which lacked GTP. Initial velocities were expressed as change in optical density per minute. The final concentrations of reagents were L-aspartate (0.5 mM), magnesium chloride (5 mM), and GTP (0.1 mM). IMP concentrations used in inhibition studies varied between 0.05 and 1.0 mM. For routine assay of adenylosuccinate synthetase a concentration of 0.25 mM was used. At this concentration the low pH fraction II gave an increase in optical density of 0.0115/min. Most of the inhibitors absorbed strongly at the wavelength of the assay at the concentrations in which it was necessary to employ them. This caused a marked reduction in accuracy due to the resulting high noise level in the spectrophotometer. For this reason inhibition studies with adenylosuccinate synthetase are reported throughout in tabular form rather than as Lineweaver–Burk plots.

Inhibition Studies. For pure noncompetitive inhibition K_i was determined from the intercept on the vertical axis of the Lineweaver–Burk plots which was taken as equal to $(1+i/K_i)/V_{max}$, where i is the inhibitor concentration.

Methods for Chemical Syntheses. Paper chromatography was carried out on Whatman No. 1 paper by the ascending method. Solvent systems were (A) isoamyl alcohol–5% aqueous disodium hydrogen phosphate (1:1) and (B) saturated aqueous ammonium sulfate–1 M, pH 6.0, potassium phosphate buffer–2-propanol (79:19:2); R_F values are given in Table I. Compounds were detected by their absorption of ultraviolet light from a Westinghouse Sterilamp G 15T8 with Corning filter no. 9863. Paper electrophoresis was carried out on Whatman No. 1 paper at a gradient of 30 v/cm in a Gelman electrophoresis chamber. Ultraviolet absorption spectra were recorded on a Cary Model 15 spectrophotometer and infrared spectra

TABLE I: Paper Chromatography of IMP Derivatives.

Compound	R_F Values in Solvent Systems A	B
Inosine 5′-chloromethylphosphonate	0.63	0.25
Inosine 5′-methylphosphonate	0.73	0.33
Inosine 5′-phosphorofluoridate	0.74	0.48
Inosine 5′-phosphite	0.75	0.45
Inosine 5′-phosphate	0.80	0.60
Inosine	0.68	0.48

[4] Grade II GTP gave slower initial rates, possibly due to inhibition by the contaminating GDP (Lieberman, 1956; Wyngaarden and Greenland, 1963).

were recorded on a Perkin-Elmer Model 137B spectrophotometer on samples in KBr disks. Proton magnetic resonance spectra were determined in deuterated dimethyl sulfoxide with a Varian A-60 spectrometer (TMS internal standard) while fluorine resonances were run in D_2O on a Varian HR 100 instrument operating at 56.4 Mcycles with hexafluorobenzene as external standard. Microanalyses were by Dr. A. Bernhardt, Mülheim, Germany, and Dr. F. Pascher, Bonn, Germany. Analytical samples were dried at 100° over phosphorus pentoxide *in vacuo* for 8 hr. Pyridine was dried over sodium hydroxide, then over calcium hydride, and distilled. Inosine 2′,3′-carbonate was prepared according to Hampton and Nichol (1966).

Results

Methylphosphonic Acid. Dimethyl methylphosphonate (17 g) was dissolved in 10 N hydrochloric acid (50 ml) and evaporated to dryness in an open vessel on a hot plate. The process was repeated three times and the crystalline residue (13 g, 98%) was dried over sodium hydroxide and phosphorus pentoxide. The product had a melting point of 100–104° (lit. mp (Hofmann, 1872) 105°). No details of this conversion appear to be available in the literature.

Inosine 5′-Methylphosphonate. Inosine 2′,3′-carbonate (0.85 g, 2.9 mmoles) was dissolved in dry pyridine (45 ml), and dicyclohexylcarbodiimide (3.5 g, 17 mmoles) and methylphosphonic acid (0.40 g, 4.2 mmoles) were added. The mixture was shaken for 5 min and after 7 hr water (20 ml) was added and the mixture was put aside overnight. Volatiles were removed under reduced pressure and the residue was slurried in water (20 ml). Dicyclohexylurea was filtered off and the filtrate was treated with 20 N ammonium hydroxide (1 ml) and heated in a boiling water bath for 5 min to hydrolyze the cyclic carbonate, then evaporated to one-half volume and applied to a 2 × 30 cm column of Dowex 1 ion-exchange resin (formate form, 8% cross-linkage). The column was eluted successively with 1 l. of each of the following solvents: 0.1 M ammonium formate–0.025 M sodium tetraborate, and 0.25, 0.5, and 1 M ammonium formate. All eluates were discarded except the last which had an optical density of 40 at 248 mμ. This eluate was evaporated to a small volume and treated with Norit (6 g) which had been deactivated by the method of Threlfall (1957). The mixture was shaken for 15 min and the Norit was filtered off. The filtrate (200 ml), which had an optical density of 4.5, was discarded. The Norit was washed with water (200 ml) and then with 5% ammonium hydroxide in 50% aqueous ethanol (200 ml). The ethanolic ammonium hydroxide washings had an optical density of about 97 and were evaporated to dryness. The residue was dissolved in methanol (10 ml) and acetone was added (30 ml). The white precipitate was centrifuged, dissolved in methanol, and reprecipitated with acetone. The solid was collected by centrifugation, washed with acetone, and dried over sodium hydroxide *in vacuo* to give the ammonium salt of inosine 5′-methylphosphonate (0.40 g, 36%) as a colorless amorphous powder. The product was chromatographically pure in systems A and B and toward electrophoresis at pH 7.4. The spectral properties in aqueous solutions were λ_{max} 253 mμ (ε 12,500) and 249 mμ (ε 11,950) at pH 12.0 and 2.0, respectively.

Anal. Calcd for $C_{11}H_{18}N_5O_7P \cdot CH_3OH$: C, 36.5; H, 5.6; N, 17.7; P, 7.8. Found: C, 37.2; H, 5.3; N, 17.1; P, 7.25.

Inosine 5′-Phosphorofluoridate. Pyridinium fluorophosphate (1.8 g, 7.6 mmoles, prepared by dissolving fluorophosphoric acid in pyridine, filtering, and drying the crystals) was dissolved in dry pyridine (10 ml) and the solution was evaporated to dryness *in vacuo*. The process was repeated and the residue was dissolved in dry pyridine (30 ml). Inosine 2′,3′-carbonate (1.0 g, 3.4 mmoles) was dissolved in the solution and dicyclohexylcarbodiimide (6 g) was added. The mixture was stirred to effect solution and stored overnight, after which a further 3 g of the carbodiimide was added. After 20 hr the solution was poured into water (40 ml) and the mixture was evaporated to dryness. The residue was slurried in 1 N ammonia (20 ml) and filtered, and the filtrate was evaporated to *ca.* 5 ml and applied to a column (2 × 25 cm) of Dowex 1 chloride. Fractions were analyzed by paper electrophoresis in 0.04 M phosphate buffer, pH 7.4. The column was first developed with 0.1 M lithium chloride (300 ml) which eluted an ultraviolet-absorbing compound with the mobility (6 cm/hr) of inosine. Elution with 0.2 M $LiCl_2$ (500 ml) removed a further trace of the same compound and subsequent elution with 0.5 M lithium chloride removed a compound with a mobility of 9.8 cm/hr. No other ultraviolet-absorbing components were detected (inosinic acid had a mobility of 14 cm/hr). The last fraction was shaken for 20 min with deactivated Norit (7 g; prepared as above). The charcoal was collected, washed with water (100 ml), and then extracted with 5% ammonium hydroxide in 50% aqueous ethanol (300 ml). The extract was evaporated to 5 ml and passed through a column of Dowex 50 in the lithium form. The eluate was clarified by centrifugation, evaporated to *ca.* 5 ml, and diluted with methanol (10 ml). Addition of acetone (40 ml) gave a white precipitate which was redissolved in 10% aqueous methanol and precipitated with acetone as before to give the lithium salt of inosine 5′-phosphorofluoridate (0.36 g, 0.87 mmole, 26%). The product was chromatographically pure in solvent systems A and B. Spectral characteristics in aqueous solutions were λ_{max} 253 mμ (ε 12,300) and 249 mμ (ε 11,900) at pH 12.0 and 2.0, respectively.

Anal. Calcd for $C_{10}H_{11}FLiN_4O_7P \cdot H_2O$: C, 32.1; H, 3.5; N, 15.0; P, 8.3. Found: C, 31.8; H, 3.65; N, 14.5; P, 8.5.

Fluorine analyses of this compound were influenced by the presence of phosphorus and varied over a wide range with repeated determinations. The fluorine magnetic resonance spectrum was typical of one fluorine atom attached to pentavalent phosphorus (Pople

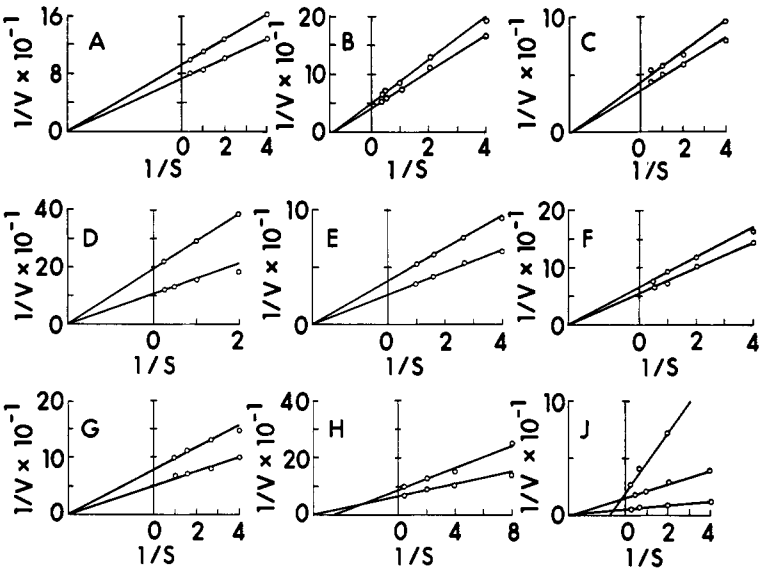


FIGURE 1: Inhibition of IMP dehydrogenase. The reciprocal of initial veloity (optical density change per minute) is plotted against one-tenth the reciprocal of the millimolar concentration of IMP. Inhibitor concentrations are given in Table II. Frame A is inhibition by inosine; B, inosine and phosphate; C, inosine and phosphite; D, inosine 5′-chloromethylphosphonate; E, inosine 5′-methylphosphonate; F, inosine 5′-phosphite; G, inosine 5′-phosphorofluoridate; H, 0.1 M phosphate; and J, 0.01 and 0.004 M ribose 5-phosphate.

et al., 1959) and lacked peaks corresponding to carbon–fluorine bonds. The peak was a doublet centered at 4494.6 cycles/sec with J = 75.5 cycles/sec. The proton magnetic resonance (pmr) spectrum showed peaks with τ values of 6.69 (H-5′), 3.98 (doublet, J = 4.8; H-1′), 1.93 (H-2), and 1.79 (H-8), and a complex of peaks in the region 5.3–6.0. The assignments were made on the basis of those of Jardetzky and Jardetzky (1960). The infrared spectrum showed a broad peak of wavenumber 840 cm^{-1} which probably corresponds to fluorine bound to pentavalent phosphorus (Bellamy, 1962) and was not produced by inosine 5′-phosphate or inosine 5′-phosphite.

Inosine 5′-Phosphite. This compound was prepared from phosphorous acid (0.27 g, 3.29 mmoles) and inosine 2′,3′-carbonate (0.5 g, 1.70 mmoles) exactly as for inosine 5′-phosphorofluoridate. Lithium inosine 5′-phosphite (0.10 g, 0.27 mmole, 16%) was obtained as a white amorphous precipitate by addition of acetone to a methanol solution. The infrared spectrum showed a weak peak of wavenumber 2390 cm^{-1} corresponding to P–H absorption (Bellamy, 1962) which was not observed in inosine 5′-phosphate. The preparation was homogeneous on chromatography in both solvent systems and on electrophoresis in 0.04 M phosphate buffer, pH 7.4, in which it had a mobility of 10.4 cm/hr.

Anal. Calcd for $C_{10}H_{12}LiN_4O_7P \cdot CH_3OH$: C, 35.7; H, 4.3; N, 15.1; P, 8.4. Found: C, 35.4; H, 4.4; N, 15.7; P, 7.9.

Studies with IMP Dehydrogenase. All the inhibitors tested on inosinic acid dehydrogenase showed pure noncompetitive kinetics with the exception of inorganic phosphate and ribose 5-phosphate which both showed mixed kinetics. The inhibitor constants are given in Table II and Lineweaver–Burk plots are shown in Figure 1. All assays were carried out in 0.025 M Tris-citrate buffer, pH 8.5, unless otherwise stated. Inhibition by inorganic phosphate was not influenced by 5 mM inosine (Table III).

Inorganic phosphite had no inhibitory effect on fresh enzyme preparations in concentrations as high as 0.15 M. With an enzyme preparation which had lost activity to the point where K_m for IMP was 3.66×10^{-3} M and V_{max} was 1.18×10^{-2} ODU/min, 0.125 M phosphite restored the original activity to give a K_m of 1.02×10^{-5} M and V_{max} of 1.76×10^{-2} ODU/min. With 0.025 mM IMP, for example, the increase in activity in the presence of the 0.125 M phosphite was 255%.

TABLE II: Inhibition of IMP Dehydrogenase.

Inhibitor	Inhibitor Concn (M)	$K_i \times 10^2$ M
Inosine	0.02	5.67
Inosine in 0.125 M phosphate	0.02	8.89
Inosine in 0.125 M phosphite	0.015	8.85
Inosine 5′-chloromethylphosphonate	0.004	0.48
Inosine 5′-methylphosphonate	0.02	4.51
Inosine 5′-phosphite	0.02	7.70
Inosine 5′-phosphorofluoridate	0.02	4.07

TABLE III: Effect of Inosine on the Inhibition of IMP Dehydrogenase by Inorganic Phosphate.

Phosphate Concn (mM)	Reduction in Rate (%)	
	No Inosine	5 mM Inosine
10	6.5	–
20	11.2	9.7
50	21.7	19.7
100	34.2	33.4

Studies with Adenylosuccinate Synthetase. The effects of inhibitors on this enzyme are summarized in Table IV. The figures show that with the exception of AMP the degree of inhibition was in all cases independent of the concentration of IMP. AMP has been shown by Wyngaarden and Greenland (1963) to be a competitive inhibitor of this enzyme. Most of the assays showed a slight increase in inhibition with increasing IMP concentration. Ribose 5-phosphate was not inhibitory at a level which strongly inhibited IMP dehydrogenase.

Attempted Detection of Progressive Enzyme Inhibition. Inosine 5′-chloromethylphosphonate, inosine 5′-phosphite, and inosine 5′-phosphorofluoridate at a level of 1 mM were incubated with adenylosuccinate synthetase in the assay buffer at room temperature for 24 hr. The loss of activity (*ca.* 20%) was in all three cases no greater than that of the controls. Paper electrophoresis indicated that no decomposition of the IMP analogs occurred during these incubations. Inosine 5′-phosphorofluoridate likewise had no effect on the rate of loss of activity when incubated with the enzyme in the presence of magnesium chloride and each in turn of the substrates IMP, GTP, and L-aspartate. Incubation of the enzyme at 40° for 5 min with 1 mM inosine 5′-phosphorofluoridate caused a loss of activity of 15% which was identical with the loss of activity in the absence of the inhibitor. The enzyme lost all activity when heated at 50° for 5 min. The enzyme was incubated with inosine 5′-phosphorofluoridate in the presence of agents expected to cause conformational changes in the protein. The losses in activity were no greater than that of the controls (Table V).

The synthetase preparation (0.1 ml) was preincubated overnight with 0.1 ml of 0.02 M solutions (pH 8) of potassium chloromethylphosphonate and fluorophosphate, respectively. No loss of activity greater than that of controls preincubated with water occurred.

IMP dehydrogenase was exposed for 1 hr to a 4.8 mM solution of inosine 5′-chloromethylphosphonate in the assay system lacking IMP and NAD. The inhibition remained constant (38 ± 2% in 4 determinations; inhibitor concentration during assay was 3.8 mM). Treatment of IMP dehydrogenase under the same conditions for 5 hr with a 1 mM solution of inosine 5′-phosphorofluoridate likewise produced no detectable progressive inhibition.

Tests for Substrate Activity. Analogs were tested at a concentration of 500 μM. The optical density at 340 mμ in the case of IMP dehydrogenase or at 280 mμ in the case of adenylosuccinate synthetase was observed for 15 min. No detectable increase in absorption occurred with any of the analogs. Under these conditions a reaction occurring at 1% the rate produced by 100 μM IMP could have been detected.

AMP Deaminase. AMP deaminase from rabbit muscle (0.285 ml) was diluted to 2 ml with 1.0 M KCl and this solution was assayed by adding 0.1 ml to 1.0 ml of a solution of 1.5 mg of AMP in 100 ml of 0.01 M citrate buffer, pH 6.5; 0.1 ml of water was added and the decrease in absorption at 265 mμ observed for 10 min. In inhibition studies water was replaced by inhibitor solution. Inosine 5′-chloromethylphosphonate added immediately prior to assay did not inhibit

TABLE IV: Inhibition of the Conversion of IMP to Adenylosuccinate by Adenylosuccinate Synthetase.

Inhibitor	Concn (mM)	Reduction in Rate (%)			
		Concentration of IMP (mM)			
		0.05	0.1	0.25	1.0
AMP	0.1	26	7	2	
Inosine	1		42	43	47
Inosine in 0.05 M phosphate	1	37	40	43	
Inosine 5′-phosphite	1		13	12	18
Inosine 5′-phosphite at pH 5.5	1		40	42	45
Inosine 5′-phosphorofluoridate	1	41	45	41	47
Inosine 5′-chloromethylphosphonate	1		45	52	53
Inosine 5′-methylphosphonate	1		50	53	57
Phosphate	150	83	80	77	
Ribose 5-phosphate	4	0	0	0	

TABLE V: Effect on Adenylosuccinate Synthetase of Potential Conformation-Changing Agents.

Agent	Concn	Reduction in Enzyme Activity (%) At Zero Time	After 24 hr
Sodium lauryl sulfate	0.25 mM	74	100
Urea	1 M	48	71
Ethylene glycol	25%	10	30

TABLE VI: Inhibition of Phosphodiesterases by IMP Analogs (0.5 mM).

Enzyme	Initial Inhibition (%) II,[a] R = F	II,[a] R = CH_2Cl	Inhibitor Concn during 20-hr Preincubn (mM)
Venom diesterase[b]	—	29[e]	2.3
Spleen diesterase[c]	64	32	0.9
Pancreatic ribonuclease[d]	0	0	3.3

[a] Scheme I. [b] Assay of Koerner and Sinsheimer (1957). [c] Assay of Hilmoe (1961). [d] Assay of Kalnitsky *et al.* (1959). [e] Competitive inhibition, $K_i = 14 \times 10^{-3}$ M, and K_m for di-*p*-nitrophenylphosphate $= 7.1 \times 10^{-3}$ M. Kinetics of the inhibitions of spleen diesterase were not examined.

detectably at a level of 834 μM. After preincubation of 0.1 ml of 0.01 M inosine 5′-chloromethylphosphonate with 0.1 ml of the enzyme solution for 24 hr no detectable inhibition occurred. Inosine 5′-phosphorofluoridate under the above two sets of conditions gave 18% inhibition either with or without the preincubation.

Studies with Phosphodiesterases. Inosine 5′-phosphorofluoridate and 5′-chloromethylphosphonate (50 μl of 10 mM solutions) were unaffected by treatment for 20 hr with bovine spleen diesterase or pancreatic ribonuclease A (0.1 ml in each case). With venom (*Crotalus adamenteus*) diesterase under the same conditions, *ca.* 50% of the phosphorofluoridate was converted to IMP and the chloromethyl analog was unchanged. In the presence of 0.1 M magnesium acetate the above conversion of phosphorofluoridate to IMP was quantitative. All the solutions were analyzed by paper electrophoresis at pH 7.4. Inosine 5′-phosphorofluoridate and 5′-chloromethylphosphonate were weak inhibitors of three phosphodiesterases and showed no tendency to inactivate these enzymes upon preincubation (Table VI).

Discussion

Inosine 2′,3′-carbonate (I) was previously found (Hampton and Nichol, 1966) to be a suitable blocked intermediate for the conversion of inosine to IMP (II, R = OH) and to inosine 5′-chloromethylphosphonate (II, R = CH_2Cl) and was employed for the preparation of the remaining IMP analogs of the present study (II, R = H, CH_3, and F) (Scheme I). The alkali-labile carbonate group of I was particularly advantageous for the preparation of inosine 5′-phosphite since nucleoside phosphites undergo extensive hydrolysis of the phosphite ester bond under the acidic conditions required to remove the usual alkylidene sugar-protecting groups (Schofield and Todd, 1961). The highly acid-sensitive 2′,3′-*O*-ethoxymethylene group has recently been utilized for the preparation of the 5′-phosphites of uridine, thymidine, and adenosine (Holy *et al.*, 1965).

Nucleoside 5′-phosphorofluoridates of adenosine, thymidine, and uridine have been prepared in good yield from the respective nucleoside 5′-phosphates and 2,4-dinitrofluorobenzene (Wittmann, 1963). In an alternative approach, inosine 2′,3′-carbonate was condensed with fluorophosphoric acid in the presence of dicyclohexylcarbodiimide to give inosine 5′-phosphorofluoridate (II, R = F) in 25% yield; optimum conditions for the conversion were not studied. The structure of the IMP analogs (II) was confirmed by their electrophoretic mobility relative to that of inosine and IMP. This indicated that each analog possessed only one ionizable group in addition to that of the cyclic amide system of the heterocyclic ring. For I (R = F) further structural confirmation was afforded by infrared, fluorine nuclear magnetic resonance (nmr), and pmr spectroscopy which established the presence of a single fluorine attached to pentavalent phosphorus and the absence of C–F bonds.

Inosine 5′-chloromethylphosphonate, although an analog of IMP with potential alkylating action, did not inactivate IMP dehydrogenase or adenylosuccinate synthetase and toward both enzymes was a weak noncompetitive inhibitor with respect to IMP. The apparent lack of affinity for the IMP site cannot be due to the large size of the chloromethyl group (van der Waals radius, 3.3 A)[5] relative to the hydroxyl (radius, 1.9 A)[5] for which it substitutes in IMP, since inosine 5′-methylphosphonate (radius of methyl, 2.0 A; Pauling, 1960), inosine 5′-phosphorofluoridate (fluorine radius, 1.4 A), and inosine 5′-phosphite (hydrogen radius, 1.2) also showed simple noncompetitive inhibition of both enzymes. Furthermore, inhibition by the phosphonates tended to increase with

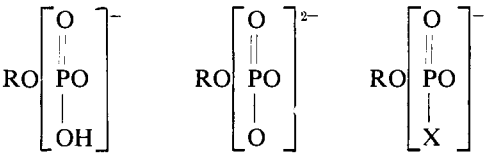
[5] Calculated from the appropriate van der Waals and covalent radii.

increasing bulk of the group attached to phosphorus. Thus, the chloromethyl analog of IMP inhibited IMP dehydrogenase ten times more strongly than did the methyl analog, and inosine 5′-phosphite inhibited adenylosuccinate synthetase to a lesser extent than did the methyl or chloromethyl analogs of IMP. Inosine itself was also a pure noncompetitive inhibitor of both enzymes and had a potency similar to that of inosine 5′-methylphosphonate. The above evidence for lack of affinity for the IMP site is in harmony with the inability of inosine to function as a substrate for IMP dehydrogenase (Magasanik *et al.*, 1957) or adenylosuccinate synthetase (Lieberman, 1956) and the lack of substrate properties among the present series of IMP analogs.

Phosphomonoester binding sites of some enzymes, *e.g.*, fructose 1,6-diphosphate aldolase (Ginsberg and Mehler, 1966), bind inorganic phosphate strongly. Evidence for such binding was not obtained for adenylosuccinate synthetase, inhibition of which by inorganic phosphate was weak and noncompetitive with respect to IMP. Ribose 5-phosphate, also, was not inhibitory to this enzyme at a concentration 100-fold higher than the K_m of IMP. With IMP dehydrogenase, a concentration of ribose 5-phosphate 200-fold higher than the K_m of IMP produced noncompetitive inhibition; a 500-fold excess of ribose 5-phosphate, however, exerted a partly competitive type of inhibition (Figure 1). Inorganic phosphate was a much weaker inhibitor of IMP dehydrogenase and at a level 5000-fold greater than the K_m of IMP gave rise to a mixed type of inhibition (Figure 1). On the other hand, the 6-thio analog of IMP, which is a substrate of IMP dehydrogenase (Hampton, 1963), does show purely competitive kinetics with respect to IMP (Hampton and Nomura, 1967); also, Wyngaarden and Greenland (1963) have shown that adenylosuccinate synthetase can be subject to simple competitive inhibition by ribonucleotides such as AMP and GMP which are close structural analogs of IMP.

The above findings suggest that binding of IMP to these enzymes is accompanied by changes in enzyme configuration mediated by the phosphate portion and possibly also by the heterocyclic portion of the IMP molecule. The present enzymes may be contrasted with 5′-nucleotidase for which ribose 5-phosphate is a strong competitive inhibitor but not a substrate and for which a conformational change caused by the heterocyclic ring of a nucleotide may be necessary for catalytic action (Koshland, 1959). One consequence of an IMP-induced binding site for IMP in the enzymes of the present study could be that inhibition by inosine might become competitive upon addition of inorganic phosphate. However, the inhibition remained noncompetitive in the presence of high concentrations of phosphate or of inorganic phosphite (which would offer less steric hindrance than phosphate to juxtaposition with inosine on the enzyme). In addition, the degree of inhibition by inorganic phosphate was not greatly influenced by the presence of inosine.

The 6-chloro analog of IMP at low concentration (one-tenth the K_m of IMP) rapidly inactivates IMP dehydrogenase; evidence that this action takes place at the IMP binding site has been discussed previously (Hampton, 1963; Hampton and Nomura, 1967). The 6-chloro analog of inosine, if indeed it acts at the same locus, is at least 200-fold less effective than its 5′-phosphate, and this accords with the other lines of evidence that inosine has little affinity for the IMP site. The pronounced reactivity of the 6-chloro analog of IMP toward the IMP site and the poor affinity of ribose 5-phosphate for that site suggest that the unsubstituted purine ring may promote binding. The oxygen at C-6 of the purine ring of IMP probably makes an additional contribution to binding (Hampton, 1963). The phosphate group of IMP is presumably bound to the enzymes as either the monoanion (III) or the dianion (IV) since the pK_a values of these species are 1.5 and 6.5, respectively, and the present studies were carried out at pH 8.



III IV V

R = inosine 5′-

The IMP analogs exist at pH 8 almost exclusively as the monoanion V. The second ionizable hydroxyl of III is essential for specific binding of IMP since even substitution by hydrogen (to give inosine phosphite) abolishes affinity for the IMP site. The present work suggests that the oxygen of the hydroxyl of the phosphomonoanion (III) does not hydrogen bond to a hydrogen contributed by the enzymes, because fluorine can form stronger hydrogen bonds than oxygen (Pimentel and McClellan, 1960) and the analog V (X = F) is noncompetitive toward IMP. The hydrogen of III might participate in binding of IMP to the enzymes; X-ray diffraction data indicate that the P–O–H system of aqueous phosphoric acid is extensively hydrogen bonded (Bastiansen and Finbak, 1944). It is conceivable that a hydrogen of the chloromethyl group of V (X = CH_2Cl) could also participate in hydrogen bonding, as does the C–H system of chloroform (Huggins *et al.*, 1955), but that the bulky chloromethyl group hinders access to the IMP site.

The hydroxyl of III is unlikely to function as a leaving group for nucleophilic attack on phosphorus by a group of the enzymes, since V (X = F) would be expected to participate more readily in such a reaction but is not a substrate for the enzymes. The oxygen of the hydroxyl of III might form a covalent bond with the enzyme, *e.g.*, an anhydride linkage to a carboxyl. However a reaction of that type would tend to occur most readily were the hydroxyl of III already ionized, *i.e.*, with IV. The present information thus tends to indicate that binding of III may involve a bond from the hy-

droxyl hydrogen to the enzyme together with an electrostatic or covalent linkage between an ionized oxygen and the enzyme.

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