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Polyamine–Polyphosphate Complexes as Enzyme Inhibitors†

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ABSTRACT: By use of the anion-exchange resin method, the apparent complex formation constants of Mg^{2+} and three polyamines with 5-phosphoribosyl 1-pyrophosphate (PRibPP) and 2,3-diphosphoglyceric acid (2,3-DPG) have been obtained. Binding of polyamines to PRibPP seems to have protected the compound from nonenzymatic hydrolysis. Analysis of the polyamine inhibition data has shown that both PRibPP– and 2,3-DPG–polyamine complexes themselves are inhibitors for enzymes. The PRibPP–spermine complex is a competitive inhibitor for hypoxanthine phosphoribosyltransferase (HPRTase, EC 2.4.2.8), and the 2,3-DPG–spermine complex is an uncompetitive inhibitor for 2,3-DPG phosphatase. Evaluation of the interaction of polyamines with PRibPP suggests that the effects of polyamines on PRibPP metabolism are complex. The compounds interact with PRibPP to form stable competitive inhibitors of many enzymes that utilize Mg^{2+}–PRibPP. The effectiveness of polyamine inhibition is a function of the Mg^{2+} concentration. In the absence of PRibPP, polyamine binding decreases the heat stability of adenine phosphoribosyltransferase (APRTase, EC 2.4.2.7) but protects PRibPP synthetase (EC 2.7.6.1) against thermal inactivation and changes the gel filtration pattern of the latter enzyme. The activity of the synthetase can be inhibited, activated, or not affected by polyamines depending upon assay conditions. Polyamines also interact with purine nucleotides and consequently change the apparent end product inhibition produced by these compounds on PRibPP synthetase and glutamine PRibPP amidotransferase (EC 2.4.2.14). ADP or 2,3-DPG can partially reverse the spermine inhibition of PRibPP synthetase. Spermine on the other hand affects feedback inhibition of amidotransferase. The polyamine inhibition constants for some of the PRibPP-utilizing enzymes have been calculated.

The polyamines are widely distributed in biological systems. Due to their polycationic nature, they not only bind to the highly negative nucleic acids but also form stable complexes with a number of polyphosphate compounds and affect many enzyme reactions (Nakai & Glinsmann, 1977a; Lovgren et al., 1978; Usui et al., 1978; Liang et al., 1979; Queigley et al., 1978; Killilea et al., 1978). However, no detailed analysis of the mechanism of polyamine effects on substrate binding has been performed. A general nonspecific substrate deprivation mechanism was assumed to be responsible for the observed inhibition of many enzyme reactions. Therefore, the biological significance of substrate–polyamine complex binding to the enzyme has not yet been established.

When an inhibitor (polyamine) binds with a substrate (polyphosphate), the inhibition observed could be due to either of the following: (1) the inhibitor combines with the substrate and decreases its effective concentration or (2) the inhibitor–substrate complex is itself an inhibitor of the enzyme.

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Reiner (1969) has derived a mathematical expression to differentiate between the two types of inhibition. With two polyphosphates that are equally important but vastly different in their biological roles, 5-phosphoribosyl 1-pyrophosphate (PRibPP) and 2,3-diphosphoglyceric acid (2,3-DPG), we began to examine the effect of their binding on enzymes that metabolize these compounds respectively in search of the specificity and biological significance of such interactions.

Perturbation sites are evident in numerous reactions involved in purine and pyrimidine nucleotide metabolism. PRibPP occupies a central role in several of these steps. Evidence obtained from recent studies on PRibPP synthetase by us and others (Yip et al., 1978; Meyer & Becker, 1977) has shown that the catalytic properties of this enzyme depend on the molecular state of the protein. The regulation may be exerted through association and dissociation of the enzyme effected by changes in nucleotide and PRibPP levels. It is thus reasonable to assume that any process that would alter nucleotides to alter their effectiveness in the enzyme reactions would also affect the biological process carried on by these proteins. Polyamines have been shown to interact with nucleotides to form stable complexes (Nakai & Glinsmann, 1977a) that affect the enzyme reaction with specificity and selectivity (Nakai & Glinsmann, 1977b). The current study was to explore the binding effect of polyamines upon the activity of various enzymes that either produce or utilize PRibPP. It was further extended to investigate the direct effect of polyamines on purine-metabolizing enzymes that require Mg^{2+} for their activation.

Materials and Methods

All the nonradioactive chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Radioactive substrates were purchased from New England Nuclear, Boston, MA.

Preparation of Anion-Exchange Resin. Dowex AG 1-X2 anion-exchange resin (200–400 mesh) (Bio-Rad Laboratories) was made into the Cl^- form with 1 N HCl followed by water washing and equilibration in 100 mM Tris-HCl (pH 7.2) buffer. The treated resin was stored in the buffer at 4 °C.

Determination of PRibPP and 2,3-DPG. The amount of PRibPP was determined by its reactivity with [^{14}C]Ade in the presence of partially purified APRTase (Yip et al., 1974). The 2,3-DPG determination was essentially the same as that described for the 2,3-DPG phosphatase assay. Premeasured assay reagents are commercially available (Sigma), and the detailed assay procedure is described in Sigma Tentative Technical Bulletin No. 665.

Binding Studies of Polyamines and Mg^{2+} to Polyphosphate Compounds. The method described by Nakai & Glinsmann (1977a) was followed with slight modification. The test mixture contained 50 mM Tris-HCl (pH 7.2), 0.1 mM PRibPP or 0.2 mM 2,3-DPG, various concentrations (0.1–40 mM) of polyamines or $MgCl_2$, and 0.5 mg (by dry weight) of anion-exchange resin in a volume of 425 μL. Equilibration was completed in 30 min by constant shaking of the mixture at 37 °C in a water bath. After the removal of the resin by centrifugation, the amount of the PRibPP or 2,3-DPG remaining in solution was determined. Parallel control experiments were run in the absence of added anion-exchange resin. Experiments were always done in duplicate, including controls. Our experimental results were consistent with those of Nakai & Glinsmann (1977a). In the presence of fixed amounts of resin and polyphosphate compound, the amount of the substance not bound to the resin was proportional to the concentration of the cations present in the solution. The same equation and derivation as those of Nakai & Glinsmann (1977a) were used here to compute and interpret our experimental data, which are as follows:

$$\frac{1}{K_d} = \frac{1}{K_d^0} + \frac{K_1}{K_d^0}[M] + \frac{K_1K_2}{K_d^0}[M] + \ldots$$

$$\frac{1}{[M]}\left(\frac{K_d^0}{K_d} - 1\right) = K_1 + K_1K_2[M] + \ldots$$

K_d is an operational quantity which is determined by dividing the amount of polyphosphate bound to resin by the amount of the polyphosphate remaining in solution. [M] represents free cation concentration.

Enzyme Assays. The activity assays of APRTase, HPRTase, and PRibPP synthetase were carried out as described previously (Yip et al., 1978; Yip & Balis, 1975). 2,3-DPG phosphatase activity was determined spectrophotometrically based on the conversion of 2,3-DPG to 3-phosphoglycerate and phosphorus. The amount of phosphorus produced under saturated substrate conditions was linear with the amount of the enzyme present. The amount of phosphorus was determined by the colorimetric reduction reaction of Fiske & Subbarrow (1925). The reaction mixture contained 1 M Tris-HCl, pH 8.0, 5 mM Mg^{2+}, 200 μg of phosphoglycolic acid, and 3–5 units of phosphoglycerate mutase (from rabbit muscle, Sigma) in a total volume of 625 μL. The reaction proceeded at 37 °C in a shaker bath for 10 min. The reaction was terminated by immediately immersing the reaction tubes in a CO_2–ethanol bath followed by addition of 0.4 mL of 8% Cl_3AcOH. The amount of phosphorus thus produced was determined by the addition of 0.2 mL of acid molybdate solution [1.25% $(NH_4)_2Mo_7O_{24}\cdot 4H_2O$ in 2.5 N H_2SO_4] and 0.05 mL of the Fiske & Subbarrow (1925) Reducer. After 10 min at room temperature, all tubes were read at 660 nm. Blanks contained everything except phosphoglycolic acid. Orotidylate pyrophosphorylase (O-5-MP) activity was determined in a mixture of glycylglycine buffer with $MgCl_2$ (0.025 M glycylglycine, pH 8.0, and 1 mM $MgCl_2$) and 100 μM [^{14}C]-orotate (specific activity 3 μCi/μmol). About 0.4 milliunit of partially purified O-5-MP (from yeast, Sigma) was used in 100 μL of reaction mixture. The reaction was performed at 37 °C in a constant shaker bath for 20 min. The reaction was terminated by immediate freezing of the mixtures in an ethanol-solid CO_2 bath. The orotate monophosphate thus formed was measured. The procedure for the recovery of the monophosphate compound from DEAE-cellulose paper (Whatman DE 81) was essentially the same as that described for HPRTase (Yip & Balis, 1976), except the concentration of the ammonium formate solution used was different. By repeatedly washing with a 12 mM ammonium formate solution, orotate was found to be completely removed from the chromatography paper while all of the radioactive orotidylate remained. The radioactivity was determined in a liquid scintillation counter. PRibPP amidotransferase activity was measured according to a modified procedure described by Holmes et al. (1973). The standard assay was performed in a total volume of 100 μL of 40 mM Tris-HCl buffer, pH 8.0, containing 2.5 mM PRibPP and 2.5 mM [^{14}C]glutamine (specific activity 1.2 mCi/nmol). The radioactive glutamine solution had been passed through a Dowex 1 ion-exchange column before it was used in the assay. The [^{14}C]glutamic acid thus formed from the reaction was recovered from DEAE-cellulose paper after repeatedly washing with 0.125 mM ammonium formate solution to get rid of the unreacted glutamine.

Enzyme Purification. APRTase, HPRTase, and PRibPP synthetase were partially purified from out-dated human erythrocytes by slight modifications of procedures that have been described previously (Yip & Balis, 1975). After hemoglobin was removed from blood lysates with DEAE-Sephadex, the crude enzyme preparation (which contained all three enzymes) was concentrated by ultrafiltration and then chromatographed in the presence of ATP and Mg^{2+} on Sepharose 6B. PRibPP synthetase separated from HPRTase and APRTase and was eluted in fractions immediately after the void volume of the column. HPRTase came out slightly ahead of the remaining hemoglobin and APRTase after the hemoglobin. Both HPRTase and APRTase thus separated were further purified by DEAE-Sephadex column chromatography followed by preparative isoelective focusing in a sucrose gradient. The purified HPRTase and APRTase used in this study had specific activities in the range of 10 and 2.5 IU/mg, respectively. Taking advantage of the aggregative nature of the PRibPP synthetase molecule in the presence of ATP and Mg^{2+}, the PRibPP synthetase collected from the first Sepharose 6B column was further purified by repeating the gel filtration process on Sepharose 6B alternatively in the absence and presence of the effectors. Dithiothreitol (1 mM) was present in all of the preparation buffers. After the first chromatography in the absence of ATP and Mg^{2+}, the minor activity peak of the larger molecular weight enzyme (Yip et al., 1978) was excluded from further purification. Whenever ATP and Mg^{2+} were not present during the gel filtration, an appropriate amount of the effector solution (3 μmol of ATP and 60 μmol of Mg^{2+} per 10-mL fraction) was pipetted into the receiving fraction tubes before collection. Complete denaturation of purified PRibPP synthetase occurred within 24 h at 4 °C in the absence of such effector and protective agents. The purified PRibPP synthetase used in our study has a specific activity in the range of 5 IU/mg. The purified enzyme is stable for several months at −20 °C in the presence of dithiothreitol, ATP, and Mg^{2+}.

PRibPP amidotransferase was purified from freshly excised rat liver (20 g). The liver was homogenized in 4 volumes of ice-cold sucrose solution (0.125 M sucrose, 0.03 M KCl, 0.07 M Tris-HCl buffer, pH 8.0, 3 mM mercaptoethanol, and 0.1 mM PRibPP). The homogenate was centrifuged for 1 h at 10000*g*. The enzyme preparation was dialyzed against 250 volumes of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 mM PRibPP, followed by chromatography on a DEAE-Sephadex column with stepwise KCl elution of the enzyme. The enzyme preparation was further purified by gel filtration on a Sepharose 6B column in the presence of 0.1 mM PRibPP in 0.1 M Tris-HCl buffer, pH 8.0. The enzyme used in the assay was about 200-fold purified from the original homogenate.

Protein Determination. The method of Lowry et al. (1951) was followed.

Results

Investigation of the Interaction of PRibPP and 2,3-DPG with Polyamines and Magnesium Ion. PRibPP forms stable complexes not only with Mg^{2+} but also with all three of the naturally occurring polyamines, i.e., spermine, spermidine, and putrescine, at pH 7.2. A plot of the data obtained with PRibPP and spermine as well as spermidine was curvilinear, and the upward curvature increased with increasing polyamine concentrations. With putrescine, a straight line over increasing polyamine concentrations was observed (Figure 1A). A secondary plot of the same data (Figure 1B) produced straight lines with a positive slope for all three complexes, but the slope

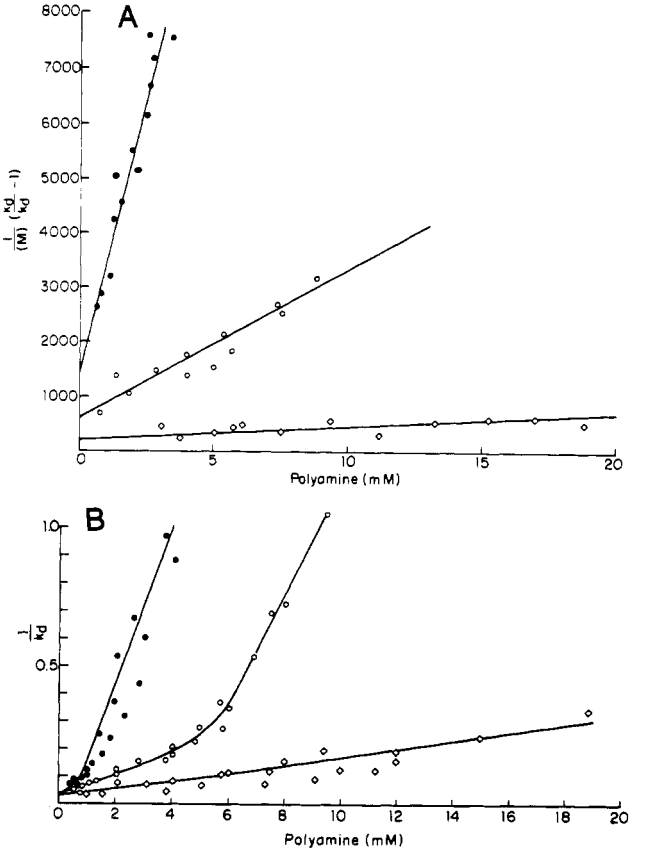

FIGURE 1: Interaction of PRibPP with naturally occurring polyamines at pH 7.2. Values of $1/K_d$ were determined by the anion-exchange resin method as described by Nakai & Glinsmann (1977a). (A) Primary plots of data obtained for PRibPP–polyamine interaction. (B) Secondary plots of the data used for (A). Values of K_d^0 were estimated from (A), and [M] represented the concentration of polyamine: (●) spermine; (○) spermidine; (◇) putrescine.

Table I: Apparent Formation Constants for Mg^{2+}- and Polyamine-PRibPP Complexes at pH 7.2[a]

constants (M^{-1})	Mg^{2+}	spermine	spermidine	putrescine
K_1	422 ± 33 (6)[b]	1812 ± 262 (4)	889 ± 99 (5)	332 ± 38 (4)
K_2	409 ± 42	547 ± 165	210 ± 35	16 ± 0.63

[a] Values of K_1 and K_2 were calculated from the secondary plots of data as described under Materials and Methods. [b] Number of determinations.

for the putrescine complex was only of borderline significance. The results indicate that two molecules of these polyamines can bind with one molecule of PRibPP. The tendency for the formation of a dication–PRibPP complex was observed to be in the decreasing order of spermine > spermidine > putrescine. A similar primary and secondary plot of Mg^{2+}–PRibPP complex has been obtained. Mg^{2+} has the greatest tendency for forming a dication salt with PRibPP (Table I).

Values of K_1 and K_2 were calculated from the secondary plots and are summarized in Table I. There is a graded increase in the apparent formation constants for complexes of PRibPP with putrescine, spermidine, and spermine. The increment seems to be related, but not directly proportional, to the number of positive charges on the polyamines. Interestingly, both spermine and spermidine show a greater tendency to bind with PRibPP than does Mg^{2+}. In the case of Mg^{2+}, the value of K_2 is very close to that of K_1 for the PRibPP complex, indicating that the affinity of free Mg^{2+} for free

Table II: Apparent Formation Constants for Mg^{2+}- and Polyamine-2,3-DPG Complexes at pH 7.2

constants (mM)	cations			
	Mg^{2+}	spermine	spermidine	putrescine
K_1	273	2654	622	115
K_2	291	763	123	81

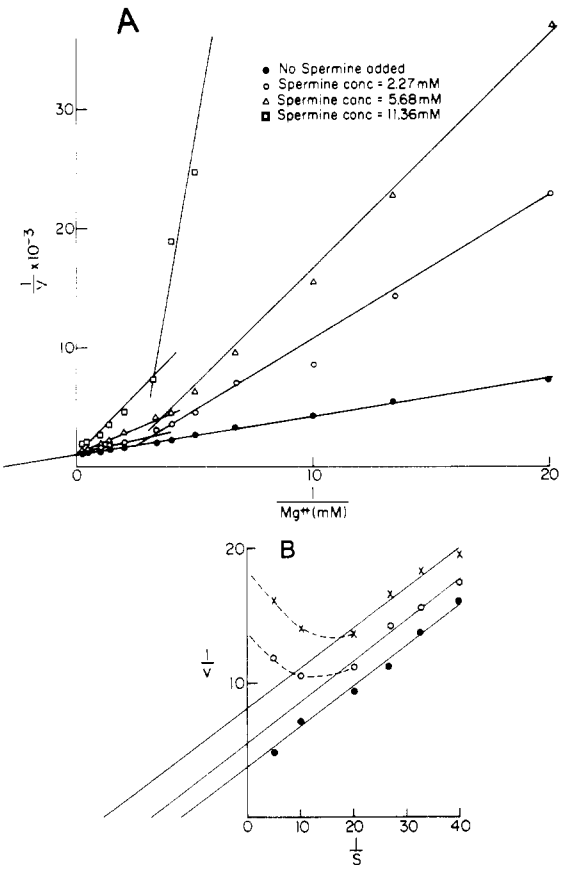

FIGURE 2: Kinetic studies of the inhibition of HPRTase and 2,3-DPG by spermine. (A) The variable concentration of the substrate in reaction mixtures at three different spermine concentrations as indicated. In the case of the HPRTase assay, Mg^{2+} concentration was the limiting factor for Mg^{2+}–PRibPP. The reaction mixture contained 0.02–0.05 μg of purified HPRTase. The reaction velocity (v) is expressed as picomoles of AMP formed per minute. In the case of the 2,3-DPG phosphatase assay (B), the double reciprocal of the initial velocity is plotted against the 2,3-DPG concentration at two different spermine concentrations: (●) control, no spermine added; (○) 2 mM spermine added; (×) 4 mM spermine added. The reaction velocity (v) is expressed as the increase in OD at 660 nm per 10 min. Assay conditions are the same as those described in the text.

PRibPP is almost the same as that for Mg^{2+}–PRibPP complex, while the affinity of free putrescine, spermidine, and spermine for their respective PRibPP complexes is only 4–30% of their affinity for free PRibPP.

Similar primary and secondary plots of polyamine– and Mg^{2+}–2,3-DPG complexes have been obtained. Values of K_1 and K_2 were calculated from the secondary plots and are summarized in Table II. The formation constant for the Mg^{2+}–2,3-DPG complex obtained by our anion-exchange resin method is very close in value to that obtained by Gupta & Benovic (1978) with their NMR studies on the same compounds.

Studies on the Polyamine Effects on the Activity of HPRTase and 2,3-DPG Phosphatase. Spermine inhibits HPRTase. The inhibition seemed to increase with decreasing concentrations of the Mg^{2+}–PRibPP complex (Figure 2A).

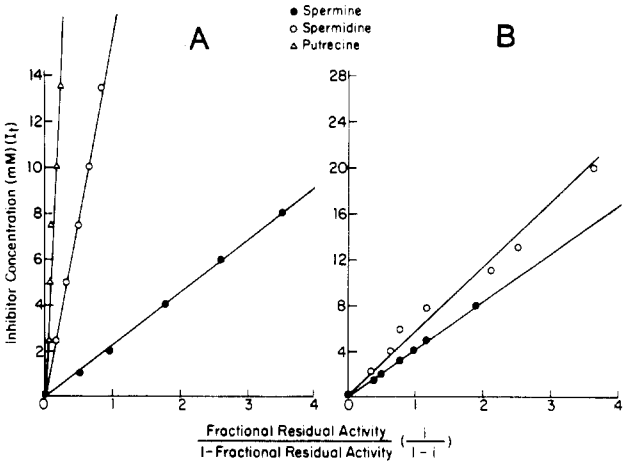

FIGURE 3: Reiner test of the polyamine complexes on enzyme inhibition. The total polyamine concentration in the assay (I_t) is plotted against the ratio of the inhibited fraction to the uninhibited fraction $[i/(1-i)]$ of the HPRTase (A) and 2,3-DPG phosphatase (B). Slopes of the lines were obtained by using a PC-100 A desktop computer programmer. Straight lines from the origin were obtained in all cases, indicating that the complexes themselves are the inhibitors. The PRibPP concentration used was 0.03 mM. The 2,3-DPG concentration used was 0.2 mM.

The result indicates a competitive pattern of inhibition of spermine on HPRTase activity. A similar observation was also made with spermidine inhibition of the same enzyme.

Spermine inhibition of 2,3-DPG phosphatase increased with increasing concentrations of the substrate (Figure 2B). Such a result indicates that the inhibition of spermine on this enzyme is more directly dependent on the amount of spermine–2,3-DPG formed in the assay and is uncompetitive in nature. The threshold amount of spermine needed for inhibition decreased with increasing concentrations of 2,3-DPG, an effect not observed with spermine inhibition of HPRTase, and was consistent with the uncompetitive inhibitory nature of the spermine–2,3-DPG complex. The same observation was true with spermidine inhibition of 2,3-DPG phosphatase.

When an inhibitor binds to a substrate, the inhibition observed can be due to (1) simple deprivation of the available substrate or (2) the substrate–inhibitor complex itself acting as an inhibitor of the enzyme. Reiner (1969) has derived a mathematical expression to differentiate between these two types of inhibition. According to Reiner, if one plots the total inhibitor concentration against the ratio of the inhibited fraction to the uninhibited fraction $[i/(1-i)]$, a hyperbolic curve is obtained, indicating the first type of inhibition. A straight line from the origin is obtained from the second type of inhibition. A straight line from the origin has been obtained from the results of the inhibition on HPRTase of the 3 polyamines (Figure 3A) and spermine and spermidine inhibition of 2,3-DPG phosphatase (Figure 3B). The inhibition by putrescine on the latter enzyme was too small to be expressed significantly. We therefore concluded from our results that the complexes produced by the three polyamines with PRibPP or 2,3-DPG are themselves inhibitors of their respective enzymes.

Studies on the Stability of Mg^{2+}- and Polyamine–PRibPP Complexes. Incubation of these complexes at 37 °C, pH 7.2 (Figure 4A), or at 55 °C (Figure 4B) destroys more of the Mg^{2+}–PRibPP complex than the others. More than 96% of the spermine–PRibPP complex was still intact after 2 h at 37 °C, as compared to 70% of the Mg^{2+}–PRibPP complex under the same conditions. The magnesium salt is less stable than even the sodium salt. The difference was more dramatic when

Table III: Inhibition Constants for Polyamines against PRibPP on Various Mg^{2+}-PRibPP-Utilizing Enzymes[a]

inhibn constants (mM)	spermine		spermidine		putrescine	
	K_i[b]	K_i'[c]	K_i	K_i'	K_i	K_i'
for HPRTase	0.95 ± 0.1	0.5 ± 0.1	8.8 ± 0.5	1.5 ± 0.2	52.5 ± 3.8	18 ± 2
for APRTase	18.3 ± 1.5	2.5 ± 0.3	47.0 ± 2.5	9.8 ± 0.7	>100.0	20 ± 1.3
for orotidylate pyrophosphorylase	22.4 ± 1.8					

[a] Values were obtained from the average of three separate determinations. [b] K_i values were obtained according to the method of Dixon & Webb (1958). The assay was performed in saturated amounts of [^{14}C]Ade and Mg^{2+}, while PRibPP concentration was the variable. [c] K_i' values are the apparent inhibition constants obtained when Mg^{2+} concentrations were lower than 0.2 mM in the assay.

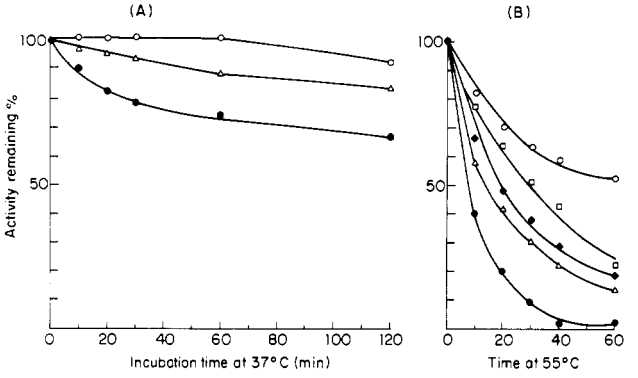

FIGURE 4: Heat stability of PRibPP–metal and –polyamine complexes. The incubation mixture contained (●) 50 μM PRibPP and 5 mM $MgCl_2$, (△) 50 μM PRibPP and 5 mM NaCl, (○) 50 μM PRibPP and 5 mM spermine, (□) 50 μM PRibPP and 5 mM spermidine, and (♦) 50 μM PRibPP and 5 mM putrescine. The amount of PRibPP that remained after incubation was determined by its APRTase reactivity in the presence of [^{14}C]Ade and excess Mg^{2+}. This value was compared with that obtained before the incubation and is expressed as the percent remaining after incubation at (A) 37 and (B) 55 °C.

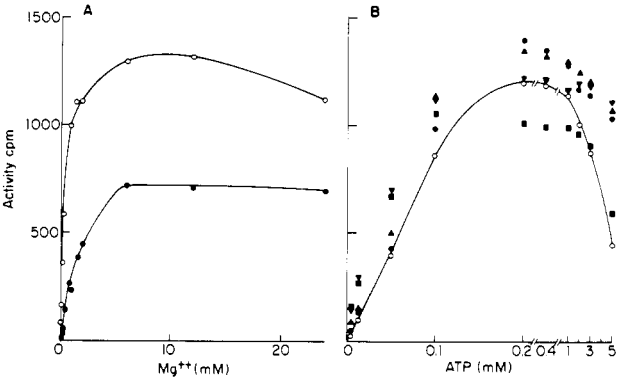

FIGURE 5: Effects of spermine on PRibPP synthetase activity in the presence or absence of a protective sulfhydryl compound. (A) 1 mM DTT was present in the reaction mixture. (B) No sulfhydryl compound was added. (○) Control, no spermine; (●) 4 mM spermine in (A), 1 mM spermine in (B); (▲) 2 mM spermine; (▼) 4 mM spermine; (■) 8 mM spermine. Experiments were all run in duplicate. Standard errors of these assays were within a 5% range.

the complexes were treated at 55 °C. After 1 h, 52% of the spermine–PRibPP complex was still intact while the Mg^{2+}–PRibPP complex was completely destroyed. The stability of the putrescine and spermidine complexes was less than that of the spermine complex.

Studies on the Polyamine Effects on the Activity of Other Enzymes. The polyamine–PRibPP complexes were found to be competitive inhibitors for many enzymes that utilize Mg^{2+}–PRibPP as substrate. A biphasic intercept of the inhibition curve was observed (Figure 2A). Two separate inhibition constants were obtained from the above data (Table III), K_i and K_i'. K_i, obtained at higher Mg^{2+} concentrations, is a competitive inhibition constant of the enzyme against Mg^{2+}–PRibPP. K_i', obtained at Mg^{2+} concentrations lower than 0.2 mM, is a competitive inhibitor constant more dependent on the concentration of Mg^{2+} than that of the Mg^{2+}–PRibPP complex. Similar observations were obtained with spermidine and putrescine on HPRTase and with the three polyamines on APRTase.

The polyamine–PRibPP complex inhibition constants for some of the purine-metabolizing enzymes are listed in Table III. These values were obtained by the method of Dixon & Webb (1958) by plotting the reciprocal of the initial velocities against various concentrations of the inhibitors at different substrate levels. All inhibition lines intercepted at one point with the uninhibited control and the K_i was thus obtained. The decrease in substrate concentration due to complex formation is too small to produce any significant effect on the intercepting point. In general, the inhibition is in the descending order of spermine > spermidine > putrescine, which is consistent with the order of their complex formation but not in any way proportional. The degree of inhibition by the spermine–PRibPP complex against various enzymes (K_i) seems to be somewhat related to the K_m value of the respective enzyme for PRibPP. For example, the HPRTase from human erythrocyte has K_m(PRibPP) = 2.4×10^{-4} M, the APRTase from the same source has K_m(PRibPP) = 6.2×10^{-5} M, and the K_m for orotidylate pyrophosphorylase from yeast = 2×10^{-5} M, and spermine is more inhibitory to HPRTase (K_i = 0.95 mM) than to APRTase (K_i = 18.3 mM) and orotidylate pyrophosphorylase (K_i = 22.4 mM). The same order of inhibition was also observed with spermidine and putrescine. When Mg^{2+} concentrations were lower than 0.2 mM, the apparent inhibition constants (K_i') for various enzymes were observed to be much lower. Putrescine, in this case, was nearly as inhibitory to APRTase as to HPRTase.

Purified PRibPP synthetase was found to be extremely sensitive to dilution and thermal inactivation (Figure 8). Dithiothreitol (DTT, 1 mM) protected the enzyme from such denaturation. We have observed that in the presence of DTT, polyamine–ATP complexes were competitive inhibitors of PRibPP synthetase with respect to Mg^{2+}–ATP (Figure 5A). The relative amount of inhibition caused by the same amount of spermine (4 mM) depended on the concentration of Mg^{2+} in the assay. However, when the enzyme was not protected by sulfhydryl compounds, stimulation of the enzyme was apparent (Figure 5B). The stimulation caused by various amounts of spermine differed with the concentration of the ATP present. The stimulation was greater above or below the substrate saturation region of the parabolic activity curve. Mercaptoethanol produced the same effect on the enzyme activity as that of DTT. However, it was found to be less effective as a preservation agent for PRibPP synthetase.

PRibPP synthetase activity is known to be inhibited by many phosphate metabolites (Fox & Kelley, 1972). In the presence of spermine, modification of such inhibitions occurred. The interaction between the two types of inhibition is most clearly shown in Figure 6; when the concentrations of 2,3-DPG and spermine were higher than 5 mM, the inhibition produced by

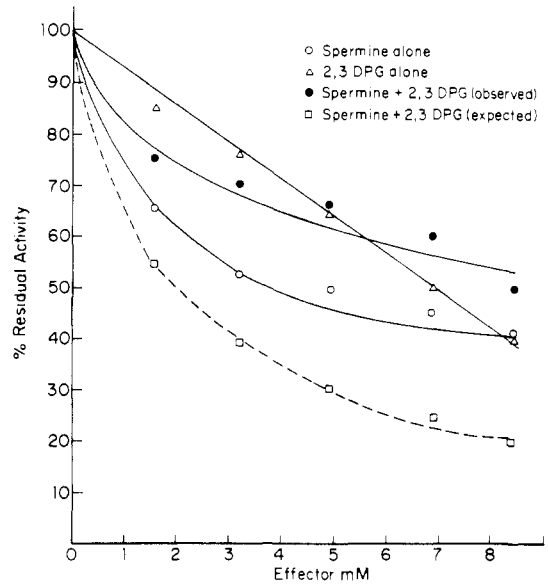

FIGURE 6: Inhibition of PRibPP synthetase by pairs of spermine and 2,3-DPG. The observed percent residual activities in the presence of various effectors (alone or in combination) are plotted in terms of the total concentration of the effector(s) present. The two inhibitors are present in equimolar concentrations. The predicted curve is determined by the product of the fractional inhibitions caused by each inhibitor alone. For example, there was 85% residual activity in the presence of 1.7 mM 2,3-DPG and 65% residual activity in the presence of the same amount of spermine. The predicted activity in the presence of 1.7 mM of both 2,3-DPG and spermine is 65 × 85% or 55%. The enzyme activities in the absence of inhibitor were 42–45 pmol of AMP formed per min. (△) 2,3-DPG alone; (○) spermine alone; (●) spermine plus 2,3-DPG; (□) activity expected when both inhibitors were present and acting independently. Assays were performed in the regular reaction mixture which contained 1 mM mercaptoethanol.

the mixture of the two was less than either alone. Similar interaction was also observed with spermine and ADP.

The inhibition of PRibPP synthetase activity by sulfhydryl blocking agents also seemed to decrease to a lesser extent in the presence of spermine.

The effects of spermine on partially purified glutamine PRibPP amidotransferase from rat liver has also been examined. Under our assay conditions, 5 mM AMP and spermine added separately produced 76 and 38% inhibition, respectively; however, when added together, a net inhibition of only 43% was observed.

Studies on the Polyamine Effects on Enzyme Stability. The heat stability of APRTase is known to be enhanced by the presence of PRibPP (Rubin et al., 1969). This protection was found to be abolished when spermine was present (Figure 7). In the absence of PRibPP, spermine made APRTase more heat labile. At the same concentration Mg^{2+} was protective in the presence of PRibPP and had no effect per se. No effect was observed with spermidine or putrescine in the presence or absence of PRibPP.

Purified PRibPP synthetase is a very labile enzyme. In the absence of phosphate ion or other protective agents, 80% of the enzyme activity was lost at 37 °C in 2 min. Spermine preserved the enzyme effectively whether or not the phosphate ion was present, but the protective effect of spermine was enhanced when the "PO_4" concentration increased (Figure 8). Mg^{2+} had no effect on the enzymes when the PO_4 was at physiological concentration or lower (parts A and B of Figure 8). ATP, as well as many other nucleotide inhibitors, protected the enzyme from denaturation. This effect was enhanced by, but did not require, the presence of Mg^{2+}. However, the stabilization by phosphate ion (at 20 mM, Figure 8C) was

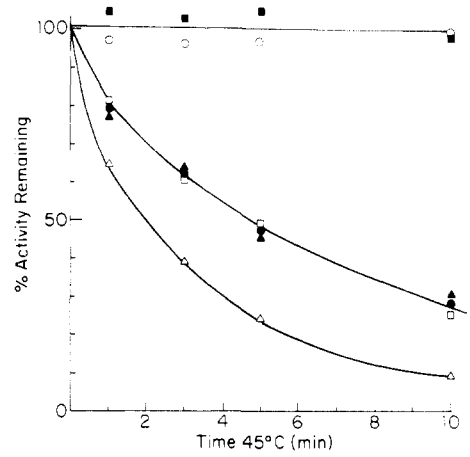

FIGURE 7: Effect of spermine on the thermal stability of APRTase. About 0.06 μg of purified APRTase was mixed with an appropriate amount of effector(s) in a total volume of 300 μL of Tris-HCl buffer, 0.1 M, pH 7.4. The mixtures were incubated in a shaker bath at 45 °C. An aliquot of 25 μL of the incubated mixture was pipetted into an ice-cold reaction mixture, which contained Mg^{2+}, PRibPP, and [^{14}C]Ade, at various times and remained in an ice–water bath. At the end of the incubation, all the treated APRTase was assayed for its activity and compared to the activity of the enzyme before incubation, which could produce ~40 pmol of AMP per min per assay. The incubation mixtures contained the following: (●) no effector added; (○) 10 μM PRibPP; (■) 10 μM PRibPP and 10 mM $MgCl_2$; (□) 10 mM $MgCl_2$; (△) 12.5 mM spermine; (▲) 12.5 mM spermine and 10 μM PRibPP.

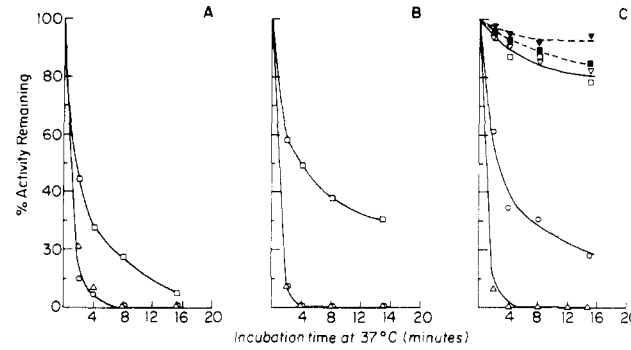

FIGURE 8: Effect of polyamines on the thermal stability of PRibPP synthetase. Less than 0.1 μg of the purified PRibPP synthetase was used in 300 μL of the incubation buffer (Tris-HCl, 0.1 M, pH 7.4), which contained no phosphate in (A), 3 mM phosphate in (B), and 20 mM phosphate in (C). The incubation procedure was the same as that described in Figure 7 except that the temperature was kept at 37 °C. The activity of the enzyme before the incubation was in the range of 42–45 pmol/(min assay). The incubation mixtures contained the following: (○) control; (□) 5 mM spermine; (△) 5 mM Mg^{2+}; (▽) 5 mM ATP; (▼) 5 mM ATP + 5 mM Mg^{2+}; (■) 5 mM ATP + 5 mM spermine.

actually eliminated when 5 mM Mg^{2+} was present. The protective effect of spermine increased slightly when ATP was present (Figure 8C). Similar studies on the effect of other spermine–nucleotide complexes have also been undertaken, and the results were essentially the same. Spermidine behaved the same as Mg^{2+} when PO_4 was absent or at a very low concentration (3 mM). At 20 mM PO_4, spermidine was effective but much less so than spermine. For example, in the presence of spermidine, incubation at 37 °C for 15 min destroyed 70% of the enzyme activity compared to 83% when no polyamine was present and 22% when spermine was present. Putrescine showed the same effects as Mg^{2+} under all the test conditions.

Studies on the Spermine Effects on the Gel Filtration Behavior of PRibPP Synthetase. Purified PRibPP synthetase

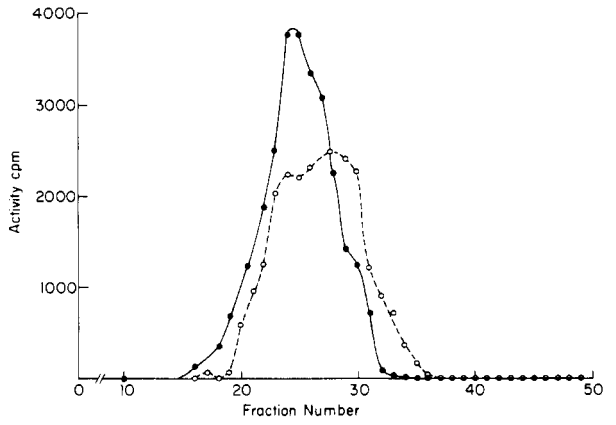

FIGURE 9: Sephadex G-150 filtration profile of purified PRibPP synthetase in the absence and presence of spermine. The column (0.9 × 30 cm) was equilibrated with a buffer containing 0.18 M KCl, 1 mM 2-mercaptoethanol, 20 mM potassium phosphate buffer (pH 7.4), and 1 mM dithiothreitol. About 0.5 μg of the purified protein in 100 μL of the buffer was used. This amount of enzyme had an activity of $\sim 5 \times 10^{-4}$ IU. All the enzyme activity was recovered after the chromatography whether in the presence (---) or absence (—) of spermine.

is very sensitive to denaturation upon gel filtration; however, this inactivation can be avoided by the presence of DTT in the running buffer. In the absence of DTT, spermine is partially protective of crude as well as purified enzyme. There was a slight shift of the enzyme activity to the later fractions upon gel filtration when spermine was present. This shift (Figure 9) was seen repeatedly upon gel filtration of both crude and purified preparations. ATP–Mg^{2+} is required to aggregate PRibPP synthetase (Yip et al., 1978). ATP–spermine was found not to be effective in this respect.

Discussion

Various workers have implicated polyamines in reactions related to cell growth and differentiation (Tabor & Tabor, 1976). However, the underlying mechanism of the polyamine effect has not been clearly shown. One source of general skepticism about the role of polyamine binding was that it might only be a nonspecific replacement of Mg^{2+} and other cations and thus diminish the amount of Mg^{2+}–nucleotide complexes. We are presenting here some evidence to show that specific inhibition patterns are apparent with different polyphosphate–polyamine complexes on different enzymes. We have found that even though PRibPP and 2,3-DPG are comparable in their capacity to form stable complexes with various polyamines, the effect of such complexing is different on their respective enzymes. We have also shown here that both PRibPP– and 2,3-DPG–polyamine complexes themselves are the inhibitors of the enzymes, and a decrease in the effective concentration of the substrate is not the sole inhibitory effect of the polyamine bindings that were seen. The effectiveness of polyamine inhibitors on HPRTase increases with decreasing concentrations of PRibPP, but their effectiveness on 2,3-DPG phosphatase is increased with increasing amounts of the substrate. The fact that the intracellular concentration of PRibPP is at the picomolar level and that of 2,3-DPG is at the millimolar level (Hershko et al., 1969; Grisolia & Joyce, 1959) seems to indicate that polyamine binding to these two polyphosphate compounds might play an effective physiological role in vivo.

The effects of polyamines are not confined to their binding to anionic metabolites. Others have shown that polyamines can affect the conformation and activity of various enzymes. Spermine stimulates the activity as well as alters the sedimentation profile of UDP-galactose 4-epimerase from yeast (Darrow & Rodstrom, 1966). In the presence of AMP, the same polyamine can facilitate the aggregation of glucogen phosphorylase monomers to an active tetramer (Wang et al., 1968). Similar activations by spermidine and other cations have been observed by Sanwal for the allosteric phosphoenolpyruvate carboxylase of *Salmonella typhimurium* (Sanwal et al., 1966) and for the glucose-6-phosphate dehygrogenase of *Escherichia coli* (Sanwal, 1970). In the latter case the activation may facilitate the production of ribonucleotide precursors essential for increased RNA synthesis. The results presented here show that spermine can alter the stability of both APRTase and PRibPP synthetase but with opposite effects. Spermine binding also changed the gel filtration pattern of the latter which suggests a possible alteration of the conformation of the enzyme.

A dual cation role for Mg^{2+} has been established in many enzyme systems which involve this ion as a catalyst (Lövgren et al., 1978). However, the function of Mg^{2+} in the PRibPP-utilizing reactions has generally been assumed to be the requirement for Mg^{2+}–PRibPP. Our results have shown that the effectiveness of polyamine inhibition is a function of the level of magnesium in the assay mixture. The biphasic nature of the Lineweaver–Burk plots of the inhibition studies strongly suggested that, at least with HPRTase and APRTase, a second free Mg^{2+} is also required for the activation of these enzymes. Polyamines compete with Mg^{2+} at both sites. Mg^{2+} binds with many phosphate metabolites to form stable complexes. The total Mg^{2+} concentration in cells has been determined (0.5–1 mM), yet the unbound Mg^{2+} might only be a small fraction of the total. The observed apparent K_i values for polyamine inhibitions are generally higher than the normal cellular level of these compounds; however, amplification of such regulatory effects under in vivo conditions as well as their manipulativity by the cellular level of free Mg^{2+} might signify a greater role of polyamines in purine metabolism than is previously known.

It has been reported that an essential sulfhydryl group is near the active site of PRibPP synthetase from rat liver (Roth & Deuel, 1974) and *S. typhimurium* (Roberts et al., 1975). The protective effect of DTT on the purified enzyme supports this. The studies of Li et al. (1978) on the role of metal ions in the mechanism of PRibPP synthetase for *S. typhimurium* have indicated that this enzyme requires two divalent cations per catalytic site. The role of Mg^{2+} is not only to supply Mg^{2+}–ATP as substrate for the enzymatic reaction but also to support the enzyme in a stereoconfiguration that is more accessible to substrates. If this is truly the case, then the Mg^{2+}–induced configuration might also have exposed the nearby essential sulfhydryl group to a more vulnerable position when the substrates are not present. The decreased thermal stability of PRibPP synthetase in the presence of Mg^{2+} is consistent with such speculation. On the other hand, spermine and spermidine with their longer side chains and extra charge groups might be able to protect the exposed sulfhydryl group by forming hydrogen bonds, therefore stabilizing as well as functionally activating the enzyme. The protective effect of spermine against the thermal and sulfhydryl group blocking inactivation on this enzyme agrees with such an argument.

PRibPP synthetase and PRibPP amidotransferase activities are subject to strong nucleotide regulation. We have noted that in the presence of both spermine and nucleotide, the inhibition observed is always less than the combined effects of the two. It seems that each acts to modulate the effect of the other.

Many current findings relate the changes in polyamine biosynthesis in cells to their proliferative activity. Recently, there have been reports showing that exposure of normal lymphocytes to phytohemagglutinin or other lectin mitogens results in an increase in the concentration of PRibPP, an accumulation of polyamines, and a highly stimulated de novo purine biosynthesis (Hovi et al., 1975; Fillingam et al., 1975; Allison et al., 1977). The results presented here seem to indicate that one of the regulatory actions of polyamines is to interact with polyphosphate compounds and modulate the existing hindrance in the de novo biosynthetic pathway caused by end product inhibition. Under certain conditions polyamines might also activate or stabilize the PRibPP synthetase and protect the integrity of its product. It is further postulated that this mechanism is not restricted to purine metabolism but may be responsible for widespread molecular control in intermediary metabolism.

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