5-Phosphoribosyl Pyrophosphate Synthetase from Ehrlich Ascites Tumor Cells*

P. C. L. Wong and A. W. Murray

ABSTRACT: In the presence of excess magnesium, 5-phosphoribosyl pyrophosphate synthetase from Ehrlich ascites tumor cells showed hyperbolic responses to increasing concentrations of adenosine triphosphate and ribose 5-phosphate; concentrations required for half-maximal rates were 0.06 and 0.05 mM, respectively. The reaction had an absolute dependence upon phosphate and half-maximal rates were obtained with a concentration of 3.3 mM. Adenosine triphosphate, deoxyadenosine triphosphate, and *O*-adenylyl methylenediphosphonate were substrates for 5-phosphoribosyl pyrophosphate synthetase; no reaction was obtained with guanosine triphosphate, deoxyguanosine triphosphate, thymidine triphosphate, cytidine triphosphate, deoxycytidine triphosphate, uridine triphosphate, xanthosine triphosphate, or inosine triphosphate. Partial inhibitions of 5-phosphoribosyl pyrophosphate synthetase were obtained with adenosine monophosphate, guanosine monophosphate, and inosine monophosphate. Adenosine monophosphate was a mixed competitive and noncompetitive inhibitor with respect to $MgATP^{2-}$; guanosine monophosphate was a noncompetitive inhibitor. Adenosine diphosphate was a competitive inhibitor with respect to adenosine triphosphate (K_i = 0.035 mM). The extracts used were contaminated with nucleoside diphosphokinase and other diphosphates were tested using *O*-adenylyl methylenediphosphonate as substrate; relatively weak inhibitions were obtained with the diphosphate of guanosine, cytidine, and uridine. Partial inhibitions of 5-phosphoribosyl pyrophosphate synthetase were obtained with several nucleoside triphosphates.

In assays containing 1 mM ribose 5-phosphate, 0.5 mM $MgATP^{2-}$, and 12.5 mM $MgCl_2$, half-maximal inhibitions were obtained with 0.25 mM cytidine triphosphate, 0.7 mM deoxycytidine triphosphate, 0.2 mM guanosine triphosphate, 0.2 mM deoxyguanosine triphosphate, 0.4 mM thymidine triphosphate, 0.4 mM inosine triphosphate, 0.4 mM xanthosine triphosphate, and 0.4 mM uridine triphosphate; maximum inhibitions obtained were 84, 48, 52, 62, 78, 66, 75, and 75%, respectively. The inhibitions by thymidine triphosphate, cytidine triphosphate, and uridine triphosphate were competitive with respect to $MgATP^{2-}$. Combinations of saturating levels of triphosphates gave inhibitions in between those obtained when the nucleotides were tested alone.

5-Phosphoribosyl pyrophosphate is a substrate for 5-phosphoribosyltransferase enzymes involved in the synthesis of histidine, tryptophan, and purine, pyrimidine, and pyridine nucleotides (for references, see Henderson and Khoo, 1965). Because PRPP is an essential precursor for a number of divergent metabolic pathways it is likely that synthesis of this compound is tightly controlled by feedback mechanisms. Switzer (1967) and Atkinson and Fall (1967) have indicated that feedback inhibition of bacterial PRPP synthetase (ATP:D-ribose 5-phosphate pyrophosphotransferase, EC 2.7.6.1) does occur and the latter workers have shown that the activity is regulated by the relative concentrations of AMP, ADP, and ATP (see also Klungsøyr *et al.*, 1968). These authors suggested that the feedback inhibition may be of the cumulative type (see Woolfolk *et al.*, 1966).

This paper describes some of the regulatory properties of PRPP synthetase partially purified from Ehrlich ascites tumor cells.

Materials and Methods

Nucleotides and Other Reagents. Nonlabeled nucleotides were obtained from the Sigma Chemical Co. and their purity was checked by chromatography in 66% (v/v) isobutyric acid adjusted to pH 4.2 with ammonia. The mono- and diphosphates used contained no detectable contaminants after chromatography in this system; GTP, dGTP, and dCTP contained 0.04, 0.01, and 0.04 molecular proportion, respectively, of the corresponding diphosphate and were not purified further. Both CTP and TTP were considerably contaminated with their diphosphates (about 0.2 molecular proportion) and were purified before use by chromatography in the isobutyric acid–ammonia system described. After drying the chromatogram, the areas containing CTP and TTP were washed with isopropyl alcohol and dried, and the nucleotides were eluted with water.

The sodium salt of ribose 5-phosphate obtained from the Sigma Chemical Co. contained no impurities detectable (Bandurski and Axelrod, 1952) after electrophoresis in 0.03 M citrate (Tris, pH 4.5).

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N-Ethylmorpholine was obtained from L. Light & Co. Ltd., Col Colnbrook, England, and redistilled before use.

ATP labeled with ^{32}P in the γ position was prepared as the *N*-ethylmorpholine salt as described by Glynn and Chappell (1964). The specific activity and purity of the preparations have been described (Murray and Wong, 1967).

[1-^{14}C]Ribose 5-phosphate was prepared from [1-^{14}C]ribose with ribokinase. Ribokinase was isolated from *Aerobacter aerogenes* as described by Horecker (1957) and catalyzed the formation of 0.21 μmole of ribose 5-phosphate/min per mg of protein at 37°. A mixture containing 8 μmoles of [1-^{14}C]ribose (specific activity 3.09 mCi/mmole), 57 μmoles of $MgCl_2$, 20 μmoles of ATP, 120 μmoles of Tris (Cl^-, pH 7.5), and ribokinase (0.42 mg of protein) in a final volume of 4.5 ml was incubated at 37° for 2 hr. Charcoal (Norit SX 2) (100 mg) was added, the mixture was filtered through Whatman No. 541 paper, the charcoal was washed with 2 ml of water, and the combined filtrate and washings were concentrated to about 1 ml. The solution was chromatographed on Whatman No. 3MM paper for 16 hr with propionic acid–1-butanol–water (50:100:70, v/v) as solvent. P_i, ribose 5-phosphate, ribose, and ATP (relative migration 1.0, 0.38, 1.60, and 0.15, respectively) were localized on the paper essentially as described by Bandurski and Axelrod (1952) and Chernick *et al.* (1951). The area corresponding to ribose 5-phosphate was washed with ethanol, dried in air, and eluted with 25 ml of water; the solution, which contained [1-^{14}C]ribose 5-phosphate, was concentrated to a volume of 10 ml. The radioactive product cochromatographed with authentic ribose 5-phosphate in the system described above and was obtained in a final yield of 30%.

[^{32}]Orthophosphoric acid was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney, and [1-^{14}C]ribose and [8-^{14}C]ATP from the Radiochemical Centre, Amersham.

Enzyme Assays. PRPP synthetase was routinely assayed with [γ-^{32}P]ATP as described by Murray and Wong (1967). Assays to test other triphosphates as pyrophosphate donors were carried out by following the incorporation of [1-^{14}C]ribose 5-phosphate into PRPP. Reaction mixtures contained 10 μmoles of *N*-ethylmorpholine (pH 8.0), 1 μmole of glutathione (neutralized with *N*-ethylmorpholine), 0.4 μmole of [1-^{14}C]ribose 5-phosphate (specific activity 0.12 μCi/μmole), 20 μmoles of phosphate (Na^+, pH 8.0), 5 μmoles of $MgCl_2$, and 0.4 μmole of triphosphate. The reactions were started with 0.05 ml of extract (about 0.2 mg of protein); the final volume of the assays was 0.4 ml. After incubating for 15 min at 37° reactions were stopped by dilution into 50 ml of ice-cold water and passed three times through two DEAE-cellulose disks held in a Millipore filter apparatus (see Atkinson and Murray, 1965). The disks were washed once with 50 ml of 5 mM NH_4HCO_3, twice with 30 ml of water, and then dried. Radioactivity associated with the disks was measured directly by liquid scintillation counting as described by Murray (1966). Addition of 2 μmoles of ATP after the dilution step did not decrease the radioactivity associated with the disks and assays using [1-^{14}C]ribose 5-phosphate and ^{32}P-labeled ATP agreed to within 6%.

Adenylate kinase activity in the enzyme preparations was measured by the formation of radioactive AMP and ADP after [8-^{14}C]ATP was equilibrated with unlabeled AMP, and nucleoside diphosphokinase by measuring the formation of [8-^{14}C]ADP after incubating [8-^{14}C]ATP and IDP as described by Murray (1968).

Results

General Properties of PRPP synthetase. The acetone-dried powders of Ehrlich ascites tumor cells used for extraction of PRPP synthetase could be stored at −15° under vacuum for at least 3 months. When stored in the presence of 10 mM glutathione or 5 mM dithiothreitol partially purified PRPP synthetase was usually stable for about 7 days at −15°; no apparent changes occurred in the catalytic or regulatory properties during this time. In the absence of thiol, all activity was lost after 4 days at −15°.

In the absence of glutathione or dithiothreitol PRPP synthetase activity was inhibited 100 and 49% by 1.0 and 0.1 mM *p*-chloromercuribenzsulfonic acid and inhibited 63% by 1 mM oxidized glutathione; the inhibitions were prevented by including 5 mM glutathione in the assays.

The partially purified extracts contained no detectable adenylate kinase activity; nucleoside diphosphokinase activity, assayed in the presence of ATP and IDP, resulted in the formation of about 25 mμmoles of ADP/min per mg of protein.

The specific activity of PRPP synthetase in the extracts used (between 20 and 80 mμmoles of PRPP formed per min per mg of protein; see Murray and Wong, 1967) was about five times that in crude extracts.

Using the ^{32}P-labeled ATP or the [1-^{14}C]ribose 5-phosphate assay, reaction rates were linear for at least 10 min; linear responses to increasing protein concentration were also obtained (up to 0.4 mg of protein/assay).

$MgATP^{2-}$ has been shown to be the true substrate of tumor cell PRPP synthetase, and concentrations of Mg^{2+} greater than the ATP concentration results in increased affinity for $MgATP^{2-}$ (Murray and Wong, 1967). In the presence of 12 mM $MgCl_2$, hyperbolic responses to increasing ATP or ribose 5-phosphate concentration were obtained; concentrations required for half-maximal rate were 0.06 and 0.05 mM, respectively. In each case Hill plots had a slope of 1.0.

The optimal pH for PRPP synthetase, measured in the presence of 1 mM ATP, of 1 mM ribose 5-phosphate, and of 12.5 mM $MgCl_2$ was about 8; rates at pH 7 and 8.6 were 76 and 98%, respectively, of the maximum rate. However, it is not possible to decide if the decrease in activity at pH values less than 8 results from a true decrease in catalytic activity. As the pH is lowered the proportion of ATP present in a fully

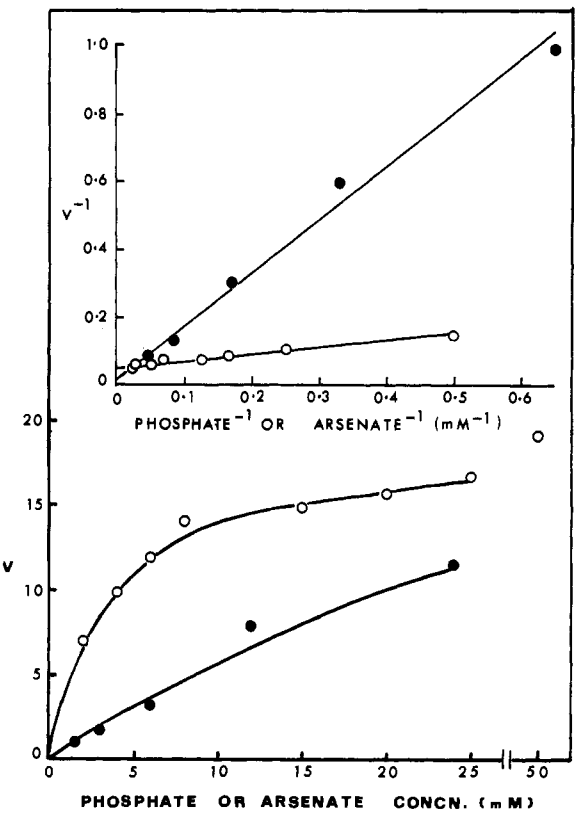

FIGURE 1: Effect of phosphate (○) and arsenate (●) concentration on the initial velocity of 5-phosphoribosyl pyrophosphate synthetase in the presence of 1 mM $MgATP^{2-}$, 1 mM ribose 5-phosphate, and 12.5 mM $MgCl_2$; v, millimicromoles of 5-phosphoribosyl pyrophosphate formed per minute per milligram of protein.

dissociated form is decreased and it can be calculated that over the pH range 7–8.6 the concentration of $MgATP^{2-}$ present in reaction mixtures containing 1 mM ATP changes from 0.5 to 0.97 mM (see O'Sullivan and Perrin, 1964). 0.5 mM $MgATP^{2-}$ may not give maximum rates at pH 7; in addition, uncomplexed ATP would also be present at pH 7 and this has been shown to inhibit PRPP synthetase (Murray and Wong, 1967).

Phosphate Requirement for PRPP Synthetase. Tumor cell PRPP synthetase has an absolute requirement for phosphate. The effect of increasing phosphate concentration on the initial velocity of PRPP synthetase in the presence of 1 mM $MgATP^{2-}$, 12.5 mM additional Mg^{2+}, and 1 mM ribose 5-phosphate is shown in Figure 1. The concentration of phosphate required for half-maximal activity was 3.3 mM (maximum velocity 20 mμmoles of PRPP formed/min per mg of protein). As shown in Figure 1 arsenate could also activate PRPP synthetase and gave half-maximal rates at a concentration of 50 mM (maximum velocity 50 mμmoles of PRPP formed/min per mg of protein). The concentrations of ATP, Mg^{2+}, and ribose 5-phosphate required to give half-maximal rates in the presence of 2 and 50 mM phosphate were also determined. Concentrations of ATP and Mg^{2+} required were the same at both levels of phosphate (0.1 and 1.45 mM, respectively); however, the concentration of ribose 5-phosphate required for 50% activity was 0.13 and about 0.02 mM in the presence of 50 and 2 mM phosphate, respectively.

Substrate Specificity. The substrate specificity of PRPP synthetase was tested using the [^{14}C]ribose 5-phosphate assay (see Materials and Methods). In the presence of 12.5 mM $MgCl_2$, 1 mM ATP or dATP gave similar rates of PRPP formation but no reaction was observed with GTP, dGTP, TTP, CTP, dCTP, UTP, XTP, or ITP. However, *O*-adenylyl methylenediphosphonate (the β,γ-methylene analog of ATP) was an effective substrate of PRPP synthetase; K_m was found to be 0.09 mM (K_m(ATP) = 0.16 mM measured with the same batch of enzyme) and the maximum velocity was 75% of that obtained with ATP. Studies on the properties of the PRPP analog formed in this reaction (5-phosphoribosyl 1-methylenediphosphonate) are in progress. The isomeric α,β-methylene analog was not a substrate for PRPP synthetase but was an effective inhibitor of the reaction. In the presence of 12.5 mM $MgCl_2$ and 0.5 mM $MgATP^{2-}$, 50% inhibition was obtained with 0.6 mM of this triphosphate analog.

Inhibition by Nucleoside Monophosphates. In the presence of 1 mM ribose 5-phosphate, of 1 mM $MgATP^{2-}$, and of 12.5 mM additional $MgCl_2$ PRPP synthetase was inhibited by AMP, GMP, and IMP; inhibition was never complete and maximum inhibitions of 95, 45, and 25% were obtained with 12 mM AMP, 5 mM GMP, and 4 mM IMP, respectively. The reaction was inhibited 50% by 0.6 mM AMP. Combinations of nucleotides gave inhibitions intermediate between those obtained with the same concentration of each nucleotide tested separately.

Under these conditions only slight inhibitions were obtained with 5 mM XMP, UMP, dUMP, and dGMP (less than 20%); 5 mM CMP, dCMP, or TMP did not significantly inhibit PRPP synthetase activity.

In assays containing 12.5 mM $MgCl_2$ AMP showed mixed competitive and noncompetitive kinetics with respect to $MgATP^{2-}$; GMP was a noncompetitive inhibitor (see Figure 2).

Inhibition by Nucleoside Diphosphates. Atkinson and Fall (1967) reported that bacterial PRPP synthetase was strongly inhibited by ADP; similar results were obtained with the tumor cell enzyme. The inhibition was competitive with respect to ATP and the value of the inhibitor constant, K_i, was 0.035 mM (K_m for ATP was 0.07 mM with the same batch of enzyme).

Partially purified PRPP synthetase used in these studies was contaminated with nucleoside diphosphokinase and diphosphates other than ADP could not be tested as regulators of enzyme activity using ATP as a substrate. Other diphosphates were tested in assays containing *O*-adenylyl methylenediphosphonate as substrate (see above); this nucleotide cannot react with nucleoside diphosphokinase. Assays were carried out by measuring the incorporation of [^{14}C]ribose 5-phosphate into PRPP (see Materials and Methods). PRPP synthetase reacting with ATP or *O*-adenylyl methylenediphosphonate was inhibited to a similar extent by

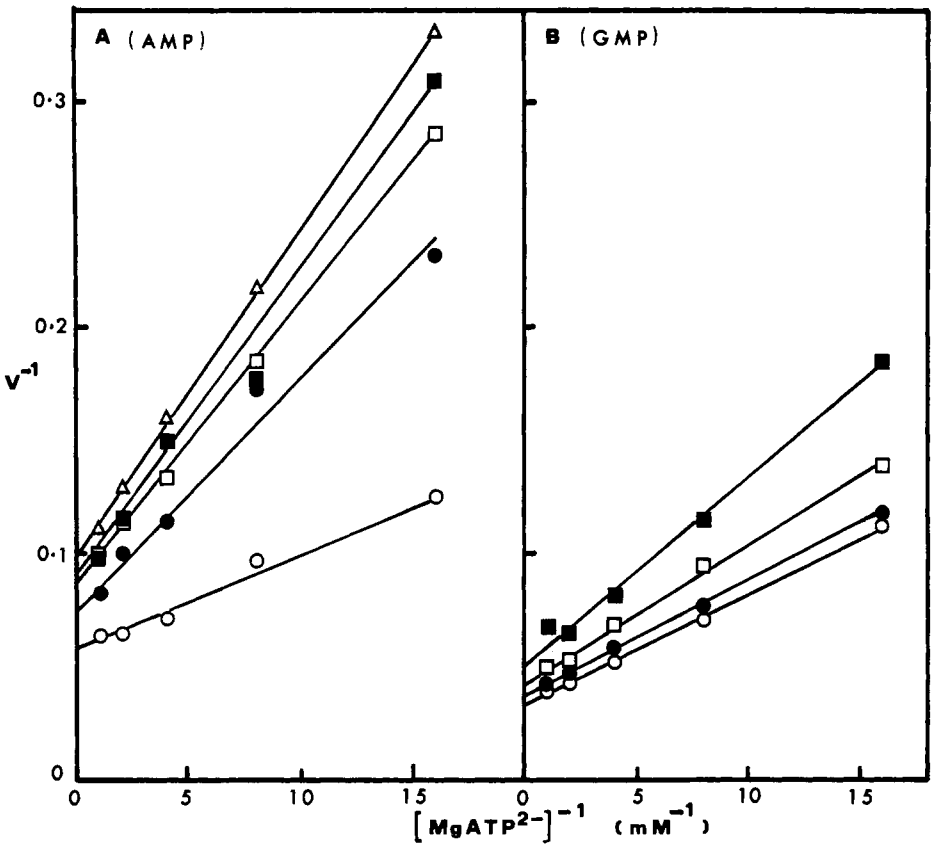

FIGURE 2: Inhibition of 5-phosphoribosyl pyrophosphate synthetase by AMP (A) and GMP (B) in the presence of 1 mM ribose 5-phosphate, 12.5 mM $MgCl_2$, and varying concentrations of $MgATP^{2-}$. AMP concentrations: 0 (○), 0.1 (●), 0.2 (□), 0.3 (■), and 0.4 mM (△). GMP concentrations: 0 (○), 1 (●), 2 (□), and 3 mM (■). *v*, millimicromoles of 5-phosphoribosyl pyrophosphate formed per minute per milligram of protein.

ADP; in assays containing 1.0 mM $MgATP^{2-}$ or 0.66 mM *O*-adenylyl methylenediphosphonate, 50% inhibition was obtained with 0.3 mM ADP. These results suggested that similar diphosphate inhibitions would be obtained with either ATP or *O*-adenylyl methylenediphosphonate as substrate. At a concentration of 2 mM CDP, GDP, or UDP inhibited PRPP synthetase by 18, 20, and 43%, respectively (*cf.* Klungsøyr *et al.*, 1968). With 0.66 mM *O*-adenylyl methylenediphosphonate as substrate, 50% inhibition was obtained with 0.33 mM adenosine methylenediphosphonate (the α,β-methylene analog of ADP).

Inhibition by Nucleoside Triphosphates. Partial inhibitions of PRPP synthetase activity were obtained with the magnesium complexes of CTP, GTP, UTP, TTP, ITP, XTP, dCTP, and dGTP (see Figure 3); assays contained 1 mM ribose 5-phosphate, 0.5 mM $MgATP^{2-}$, and 12.5 mM $MgCl_2$. Half-maximal inhibitions were obtained with 0.25 mM CTP, 0.7 mM dCTP, 0.2 mM GTP, 0.20 mM dGTP, 0.40 mM TTP, 0.4 mM ITP, 0.4 mM XTP, and 0.4 mM UTP; the maximum inhibitions obtained were 84, 48, 52, 62, 78, 66, 75, and 75%, respectively. Equimolar Mg^{2+} and triphosphate were added to assays so that concentrations of uncomplexed Mg^{2+} remained essentially constant in these experiments. The inhibitions by TTP, CTP, and UTP were competitive with respect to $MgATP^{2-}$ (Figure 4). Inhibition of PRPP synthetase by GTP did not give simple kinetics; nonlinear double reciprocal plots were obtained at GTP concentrations higher than 0.1 mM (See Figure 4). With 0.025 and 0.05 mM $MgATP^{2-}$ inhibition of enzyme activity decreased with increasing GTP concentration; similar results were obtained in four separate experiments. K_i values for inhibition by TTP, CTP, and UTP were not calculated as these values are meaningless for inhibitors showing partially competitive kinetics (see Dixon and Webb, 1964).

Except with UTP and TTP combinations of saturating concentrations of triphosphates resulted in inhibitions intermediate between the highest and lowest inhibition found when the nucleotides were tested separately. Combinations of UTP and TTP consistently gave inhibitions indicative of cumulative inhibition (Woolfolk and Stadtman, 1964).

Response of PRPP Synthetase Activity to "ATP Charge." The response of PRPP synthetase from Ehrlich ascites-tumor cells to "ATP charge" was similar to that described by Atkinson and Fall (1966) for the bacterial enzyme.

Effects of Other Metabolites on PRPP Synthetase Activity. In the presence of 12.5 mM $MgCl_2$, 0.25 mM $MgATP^{2-}$, and 1 mM ribose 5-phosphate, 1.8 mM NAD, NADH, NADP, and NADPH inhibited PRPP

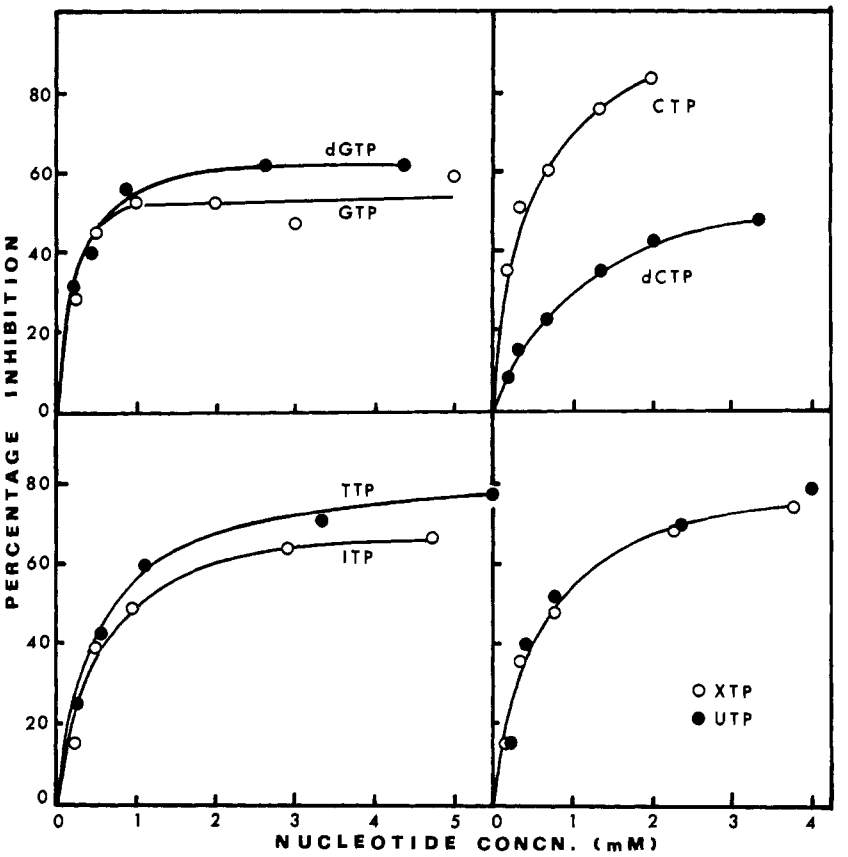

FIGURE 3: Inhibition of 5-phosphoribosyl pyrophosphate synthetase by nucleoside triphosphates. Assays contained 12.5 mM $MgCl_2$, 1 mM ribose 5-phosphate, 0.5 mM $MgATP^{2-}$, and varying concentrations of the indicated triphosphates.

synthetase by 20, 35, 20, and 20%, respectively; these inhibitions were overcome by increasing the concentration of $MgATP^{2-}$ to 1 mM. In assays containing 12.5 mM $MgCl_2$, 0.5 mM $MgATP^{2-}$, and 1 mM ribose 5-phosphate no inhibition was obtained with 5 mM L-histidine or L-tryptophan, 0.6 mM adenosine, inosine, or guanosine, and 1 mM glyceraldehyde 3-phosphate, glucose 6-phosphate, or fructose 1,6-diphosphate. Under similar conditions but with 1 mM $MgATP^{2-}$ and 0.5 mM ribose 5-phosphate PRPP synthetase was not inhibited by 1 mM PRPP.

Discussion

PRPP synthetase from Ehrlich ascites tumor cells was clearly shown to have an absolute requirement for P_i. Previous workers have provided indirect evidence for a P_i requirement of mammalian PRPP synthetase (Remy *et al.*, 1955; Kornberg *et al.*, 1955; Preiss and Handler, 1958; Hershko *et al.*, 1967); this requirement has also recently been reported for PRPP synthetase from *Salmonella typhimurium* (Switzer, 1968a,b). Switzer (1968a) has shown that in the presence of P_i and absence of ribose 5-phosphate the bacterial enzyme reacts with ATP forming an enzyme–pyrophosphate intermediate and presumably releasing AMP; elucidation of the mechanism of the P_i involvement will require studies on the binding of substrates by PRPP synthetase in the presence and absence of P_i.

Because of the extreme lability of tumor cell PRPP synthetase its purification was not undertaken further. Preliminary studies showed that the purity could be increased about twofold by precipitation between 30 and 50% ethanol; this step was not used in routine preparations of PRPP synthetase because it did not remove the contaminating nucleoside diphosphokinase activity. Despite this contamination the effects of diphosphates on PRPP synthetase could be tested using the β,γ-methylene analog of ATP, which can not react with the diphosphokinase, as substrate. The results obtained with phosphonate analogs in this study emphasize their usefulness as mimics of the catalytic and regulatory properties of natural nucleotides (for references to earlier work, see Hershey and Monro, 1966; Atkinson and Murray, 1967; Duée and Vignais, 1968). In addition it has been shown that the phosphonate analog of ADP (adenosine methylenediphosphonate) is a substrate for nucleoside diphosphokinase (M. R. Atkinson and R. T. James, unpublished data).

The findings reported in this paper have shown that a large number of end products of PRPP metabolism can inhibit PRPP synthesis but it is not clear which regulate its activity *in vivo*. However steep response curves of enzyme activity were obtained as the relative proportions of AMP, ADP, and ATP were varied and it seems likely that this may provide a relatively nonspecific control related to the energy status of the cell as described by Atkinson and Fall (1967); control

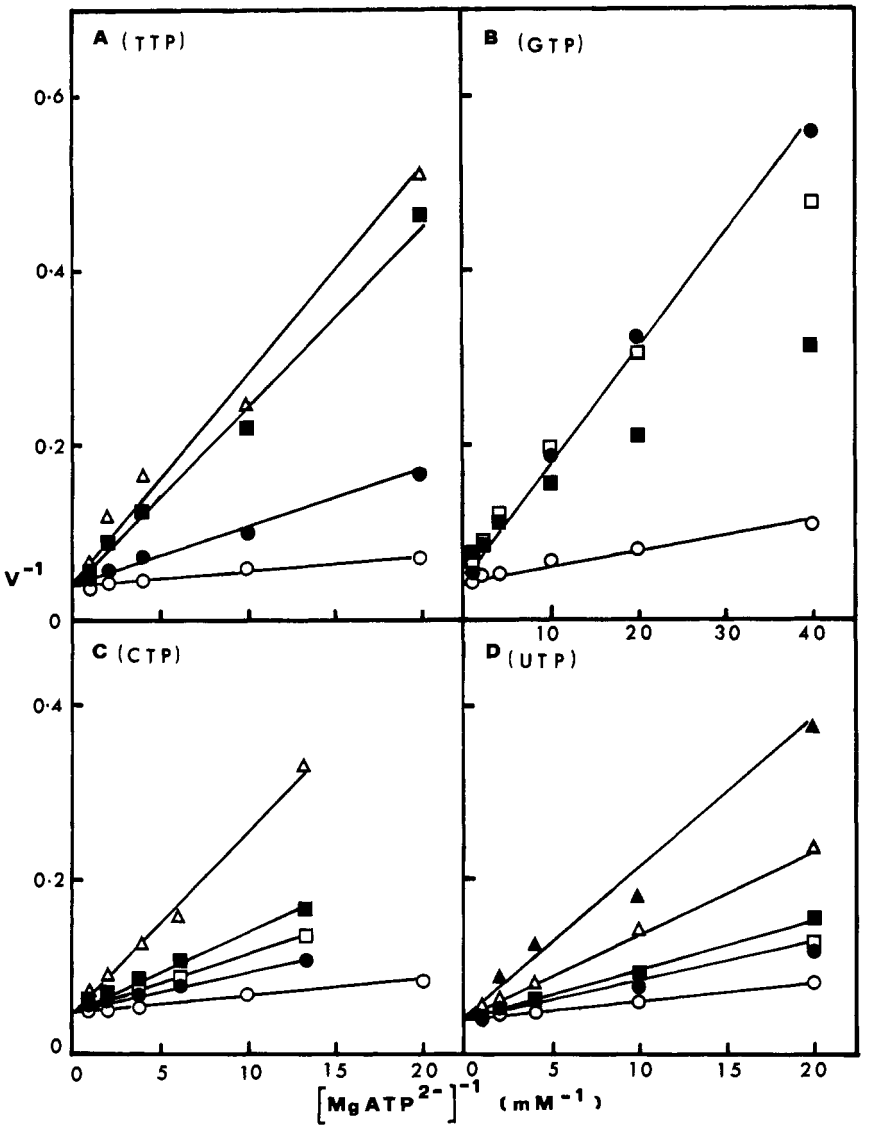

FIGURE 4: Inhibition of 5-phosphoribosyl pyrophosphate synthetase by nucleoside triphosphates. Assays contained 12.5 mM $MgCl_2$, 1 mM ribose 5-phosphate, and varying concentrations of $MgATP^{2-}$. (A) TTP concentrations: 0 (○), 0.1 (●), 0.5 (■), and 1.0 mM (△). (B) GTP concentrations: 0 (○), 0.1 (●), 0.25 (□), and 1.0 mM (■). (C) CTP concentrations: 0 (○), 0.05 (●), 0.1 (□), 0.2 (■), and 0.5 mM (△). (D) UTP concentrations: 0 (○), 0.1 (●), 0.2 (□), 0.3 (■), 0.5 (△), and 1.0 mM (▲). v, millimicromoles of 5-phosphoribosyl pyrophosphate formed per minute per milligram of protein.

related to the requirements of the different pathways utilizing PRPP would be provided by the other inhibitors, particularly nucleoside triphosphates (see also Klungsøyr *et al.*, 1968). The finite inhibitions obtained with increasing concentrations of triphosphates would prevent accumulation of any one end product from completely inhibiting PRPP synthetase. A saturating level of inhibition is usually interpreted as indicating separate binding sites for substrate and inhibitor; alternatively the enzyme preparations could contain a form of PRPP synthetase insensitive to triphosphate inhibition.

It does not seem likely that PRPP itself normally regulates the activity of PRPP synthetase in Ehrlich ascites tumor cells; in the absence of added glucose the concentration of PRPP in these cells is low (about 0.3 μmole/g of tumor cells; Henderson and Khoo, 1965) and in these experiments 1 mM PRPP did not inhibit enzyme activity. In addition the level of PRPP in Ehrlich cells *in vivo* rises when utilization of PRPP by PRPP amidotransferase is inhibited by administration of 6-methylthioinosine (Paterson and Wang, 1968); this indicates that PRPP synthesis continues with presumably no increase in the ribose 5-phosphate concentration.

Addition of glucose has been shown to give large increases in PRPP accumulation in Ehrlich ascites tumor cells *in vitro* suggesting that ribose 5-phosphate may normally be limiting for PRPP synthesis (Henderson and Khoo, 1965). Although interpretation of these workers' results is difficult as glucose administration results in depletion of the adenine nucleotide pool and changes in the relative concentrations of the adenine nucleotides (Overgaard-Hansen, 1965), it is likely that

an understanding of the regulation of PRPP synthetase will need to include studies on the control of pentose phosphate supply.

Acknowledgment

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