

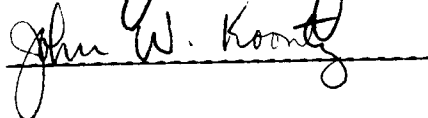
To the Graduate Council:

I am submitting herewith a dissertation written by Charles Randolph Crawford entitled, "Characterization of Mutant and Wild-Type Phosphoribosylpyrophosphate Synthetase from Chinese Hamster Fibroblasts." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.


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We have read this dissertation
and recommend its acceptance:

Accepted for the Council:


The Graduate School

CHARACTERIZATION OF MUTANT AND WILD-TYPE
PHOSPHORIBOSYLPYROPHOSPHATE
SYNTHETASE FROM CHINESE
HAMSTER FIBROBLASTS

A Dissertation
Presented for the
Doctor of Philosophy
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Charles Randolph Crawford

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ABSTRACT

A previous investigation of the clonal cell line KC62 had shown it to have significantly reduced intracellular PRPP levels when compared to the parental V-79A cell line. In an attempt to explain this phenotypic difference in the two cell lines, the physical and kinetic properties of the enzyme responsible for PRPP production, PRPP-synthetase, have been examined. Initial examination of the catalytic properties of the V-79A and KC62 PRPP-synthetase was conducted using 110,000 x G supernatant preparations of each enzyme. Determination of the kinetic parameters (K_m and V_{max}) for the V-79A and KC62 enzyme provided no evidence for a difference in their catalytic properties. Subsequent studies of the enzymatic activity in the presence of various nucleotides, however, demonstrated the KC62 enzyme to have an increased sensitivity to a variety of nucleotide inhibitors compared to its V-79A counterpart.

A scheme for the purification of V-79A and KC62 PRPP-synthetase was devised and the kinetic and physical properties of the purified enzymes were examined. K_m values for the V-79A and KC62 PRPP-synthetase were found to be very similar. Inhibition of both enzymes by AMP, GMP and XMP was examined with respect to both substrates. For any given inhibitor and variable substrate, the resulting type of inhibition was found to be the same for the V-79A and KC62 enzyme. The apparent K_i values for the KC62 enzyme, however, were in all cases less than those of the V-79A enzyme. These results suggested that the KC62 enzyme possessed a structural alteration rendering it more sensitive to inhibition by nucleotide inhibitors. Examination of the physical properties of the two enzymes failed to provide evidence of a major

structural difference between the two enzymes. KC62's subunit molecular weight, molecular weight, and pattern of thermal inactivation at 55°C was identical to that of the V-79A enzyme.

Finally, the consequences of KC62's reduced PRPP levels on its nucleotide pool sizes have been assessed. The IMP, GMP NADH and CDP pools of the KC62 cells were significantly less than those of V-79A cells. While it is reasonable to attribute KC62's reduced nucleotide pools to its reduced PRPP levels, the specific mechanisms involved have not been determined.

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CHAPTER I

INTRODUCTION

PRPP (5-phosphoribosyl-1-pyrophosphate) is an essential substrate in the biosynthetic pathways leading to purine nucleotides (1,2,3), pyrimidine nucleotides (4,5), pyridine nucleotides (6,7), and in bacteria to histidine (8,9) and tryptophan (10,11). PRPP is also required for the salvage of purines and pyrimidines by the phosphoribosylation reactions. Intracellular steady state levels of PRPP have been found to be low in all tissues tested, ranging from a low of 2 μ M in human erythrocytes (12) to a high of 15 μ M in liver extracts (13). These low intracellular PRPP levels in most cases represent concentrations substantially less than the Michaelis constant for PRPP of the PRPP utilizing enzymes in the aforementioned pathways (14,15). It has, therefore, been hypothesized that PRPP availability may be a regulatory factor in the control of these divergent synthetic pathways. Conclusive documentation of limited PRPP availability playing a crucial, regulatory role in the de novo purine and salvage pathways exists (14,16). At present, evidence for a regulatory role for PRPP is less compelling for the other PRPP-utilizing synthetic pathways. A further indication of the importance of PRPP levels are the human disorders hypouricemia, hyperuricemia, Lesch-Nyhan syndrome, and nonspherocytic hemolytic anemia; all of which can include altered PRPP levels as part of their pathology.

Because PRPP is an essential substrate for these diverse biosynthetic pathways and a significant regulatory factor in at least some cases, PRPP production and the factors regulating it are of profound biological significance.

The enzyme PRPP-synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase:E.C. 2.7.6.1) is ubiquitous in mammalian systems and catalyzes the formation of PRPP from the substrates Mg-ATP and R-5-P (ribose-5-phosphate). Previous studies of the enzyme from several sources have determined it to be a large, multimeric enzyme under physiological conditions (17,18,19,20). The enzyme from all sources tested has an absolute requirement for inorganic phosphate which acts as a positive allosteric modulator. In vivo, regulation of PRPP-synthetase activity is complex. PRPP-synthetase activity may, in part, be determined by availability of R-5-P, a product of the pentose pathways (21,22,23). Enzyme activity is also dependent on the concentration of a number of end products of the biosynthetic pathways for which PRPP is an essential substrate. These end products are thought to act as feedback inhibitors of PRPP-synthetase activity (23,24,25,26).

The biochemical investigation described in this dissertation has as its primary focus the PRPP-synthetases from the cultured, fibroblast cell line V-79A and a V-79A derived clonal line KC62. This investigation represents a continuation of a previously undertaken investigation of KC62's decreased ability to salvage exogenously supplied purine bases (27). The KC62 cell line originated from ethylmethane sulfonate-mutagenized V-79A cells which had undergone three cycles of ^3H -adenine suicide to select for cells with a decreased ability to salvage exogenously supplied adenine. KC62 cells were found to have approximately a 70% reduction in their uptake of the purine bases, adenine, hypoxanthine and guanine as compared to their V-79A parent. Factors potentially responsible for KC62's decreased salvage ability were examined including membrane transport rates and the kinetic parameters of the salvage enzymes APRT (adenine phosphoribosyltransferase) and HPRT (hypoxanthine

phosphoribosyltransferase). The results of these studies revealed no significant differences in these parameters for the two cell lines. It was subsequently discovered that KC62's steady state PRPP levels were only 30% of those of V-79A. It was concluded that KC62's reduced ability to salvage exogenously supplied purines by phosphoribosylation was the result of its reduced intracellular PRPP levels. The specific cause of reduced PRPP levels in KC62 remained to be determined. While it was possible to hypothesize numerous potential causes for KC62's reduced PRPP levels, results from the initial characterization were suggestive of an alteration in the PRPP-synthetase activity of KC62 cells.

The purpose of the work described in this dissertation was to attempt to document a difference in the kinetic and/or physical properties of the PRPP-synthetases of V-79A and KC62. Additionally, the consequences of KC62's reduced PRPP levels on the nucleotide pool sizes have been assessed.

CHAPTER II

CELLS AND GROWTH

Two cell lines, V-79A and KC62, were used in all experiments. V-79A was cloned from V-79 (28) (a Chinese hamster lung fibroblast line) by Irene Schauer (29). V-79A is a stable heteroploid line with 21 chromosomes. The clonal line KC62 was isolated from ethylmethane sulfonate-mutagenized V-79A cells by means of three cycles of ^3H -adenine suicide. Microscopic examination of KC62 revealed no gross morphological differences from V-79A, and both cell lines have a generation time of approximately 12 hours. Both cell lines were grown as monolayers in filter-sterilized LAC medium on 100 mm diameter plastic culture plates (Corning Glass Works) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . For some experiments both cell lines were grown as monolayers in LAC medium in sealed 4.3 x 11.2 inch glass roller bottles (Wheaton Scientific) at 37°C at 17 revolutions per hour. LAC medium consists of 10.6 g/liter of Hanks' Minimal Medium (Flow Laboratories, Inc.), supplemented with 10% fetal calf serum (Sterile Systems, Inc.), 20 ml/liter of 10% lactalbumin hydrosylates solution (Grand Island Biological Company), 1.05 g/liter of porcine trypsin (Grand Island Biological Company), 1.3 g/liter of sodium bicarbonate, 2 mg/liter of fungizone (Flow Laboratories, Inc.), 0.058 g/liter of L-glutamine, and 50 mg/liter of Gentamycin (Schering Corporation).

Growing cells were continuously maintained by serial culturing every two to three days. Removal of the cells from plates or roller bottles was normally accomplished with a 0.25% trypsin solution containing 8.0 g/liter of sodium chloride, 0.4 g/liter of potassium chloride, 1.0 g/liter of dextrose, 0.2 g/liter

of disodium ethylenediamine tetraacetate (EDTA), 2.5 g/liter porcine trypsin, and 1.078 g/liter of sodium bicarbonate.

CHAPTER III

V-79A AND KC62 PRPP-SYNTHETASE OF 110,000 X G SUPERNATANTS

A. Preparation of 110,000 x G Supernatants

Materials and Methods

The medium was removed from ten 100 mm culture plates which were approximately 70% confluent and the cells were trypsinized. The cells were resuspended in LAC medium (3 ml/plate), pooled, and centrifuged at 120 x G for 5 minutes. The supernatant was removed and the cell pellet was washed by centrifugation twice with 8 ml of Hanks' balanced salt solution (Flow Laboratories, Inc.). The resulting cell pellet was resuspended in 6 ml of 4°C extraction buffer (pH 7.4) consisting of 50 mM Na_3PO_4 , 6 mM MgCl_2 and 0.1 mM dithiothreitol (DTT). The cells were lysed by freezing in liquid nitrogen and thawing five times. The lysate was centrifuged at 30,000 x G for 20 minutes at 4°C. The resultant supernatant was then centrifuged at 110,000 x G for 60 minutes at 4°C. The 110,000 x G supernatant was placed in a Spectrapor 2 (Spectrum Medical Industries, Inc.) membrane and dialyzed for 12 hours in 1,600 volumes of extraction buffer at 4°C. Two hundred microliter aliquots of the dialyzed supernatant were distributed to precooled 0.5 ml microfuge tubes (Cole-Parmer Instrument Company) and stored at -70°C. The protein concentration of the dialyzed 110,000 x G supernatant was determined by the method of Lowry (20) using bovine serum albumin (Sigma Chemical Company) as a standard.

Results

The 110,000 x G supernatant was prepared by the above outlined procedure twenty-five times. The average volume recovered after dialysis was 5.3 ml with an average protein concentration of 1.8 mg/ml. The average total protein recovered was 9.5 mg with an average specific activity of 3.50 nanomoles PRPP produced/minute/mg of protein at saturating concentrations of both substrates. (See Chapter III, Initial Velocity Studies, Materials and Methods for PRPP-synthetase assay procedure.) There was no apparent difference in either the average total protein recovered or the average final specific activity between the V-79A and KC62 supernatant preparations.

B. Initial Velocity Studies

Materials and Methods

PRPP-synthetase activity in 110,000 x G supernatants was measured using a modification of the methods of Reem (31), and Prabhakararao and Jones (32). The method involves coupling the PRPP-synthetase reaction to the following reactions:

1.
$$[\text{Carboxy-}^{14}\text{C}]\text{orotic acid} + \text{PRPP} \xrightarrow{\text{OPRT}} [\text{Carboxy-}^{14}\text{C}]\text{orotidine-5'-phosphate} + \text{PPi}$$
2.
$$[\text{Carboxy-}^{14}\text{C}]\text{orotidine-5'-phosphate} \xrightarrow{\text{ODC}} \text{UMP} + {}^{14}\text{CO}_2$$

By trapping evolved ${}^{14}\text{CO}_2$ it was possible to determine PRPP production and thereby monitor PRPP-synthetase activity. Baker's yeast OPRT (orotate phosphoribosyltransferase) and ODC (Orotidine-5'-phosphate decarboxylase) were purchased from P-L Biochemicals, Inc., in a lyophilized form. The disodium salts of ATP and R-5-P were purchased from Sigma Chemical Company and

[carboxy- ^{14}C]orotic acid (51.1 mCi/mmole) was purchased from New England Nuclear.

The final assay mixture for each determination had a final volume of 500 μl and contained the following:

1. 100 μl of 0.1111 M Na_3PO_4 buffer (pH 7.4) containing 0.0133 M MgCl_2
2. 20 μl of 0.41 mM [carboxy- ^{14}C]orotic acid (0.01 $\mu\text{Ci}/\mu\text{l}$) in H_2O
3. 80 μl of 0.5 mg/80 μl OPRT-ODC in 0.1111 M Na_3PO_4 buffer (pH 7.4) containing 0.1033 M MgCl_2
4. 100 μl of 25, 50, 75, 100, 125 or 2500 μM ATP in H_2O
5. 100 μl of 25, 50, 100, 150, 200 or 2500 μM R-5-P in H_2O
6. 100 μl of diluted, dialyzed 110,000 x G supernatant (50 mM Na_3PO_4 buffer (pH 7.4) containing 6 mM MgCl_2 and 0.1 mM DTT)

The final concentrations in the assay mixture were 50 mM Na_3PO_4 , 6 mM MgCl_2 , 16.4 μM orotic acid, 20 μM DTT, 5, 10, 15, 20, 25 or 500 μM ATP and 5, 10, 20, 30, 40 or 500 μM R-5-P.

All components (at 4°C) of the assay mixture except the 110,000 x G supernatant were added to a 15.7 x 100 mm polypropylene test tube (Walter Sarstedt, Inc.) and warmed at 37°C for 10 minutes. The enzyme reaction was started by adding 100 μl of diluted 110,000 x G supernatant (which had been warmed at 37°C for 3 minutes) to the test tube containing the assay mixture. The tube was immediately capped with a rubber stopper (Kontes) fitted with a polypropylene center well (Kontes) (the stoppers are supplied with a uniform precut central hole that is just sufficient to fit the stem of a center well). The center well was approximately 4-5 cm above the reaction mixture. Prior to its use, the center well had inserted into it a 12 x 22 mm piece of Whatman

3 MM paper (folded to one third its length) which had been wetted with 70 μ l of 1.0 M KOH. The capped test tube was transferred to a shaking, 37°C water bath. After 6 minutes the reaction was stopped by injection of 200 μ l of 4 M perchloric acid into the reaction mixture through the rubber cap. The test tube was left in the same water bath for one hour to complete the adsorption of released $^{14}\text{CO}_2$ by the KOH wetted filter paper. At the end of this time the filter paper was removed from the center well and placed in a 20 ml, capped polyethylene vial containing 1 ml of H_2O and 10 ml of triton-toluene scintillation fluid. The triton-toluene scintillation fluid was made from Fisher Scientific Company's Scintiprep (25X solution containing PPO-POPOP), Triton X-100 (Sigma Chemical Company) and Scintanalyzed toluene (Fisher Scientific Company). Liquid scintillation counting was done with a Beckman LS-230 Liquid Scintillation Counter.

In each experiment a standard curve was generated by substituting PRPP standards for R-5-P in the mixture. $^{14}\text{CO}_2$ evolution was linear with respect to final PRPP concentrations up to 10 μM PRPP. Nanomoles of PRPP generated was calculated by reference to standard curves. The apparent K_m and V_{max} values were determined by analyzing the resultant data using the weighted, nonlinear regression method of Wilkinson (33).

Results

Figures 1 and 2 show double reciprocal plots of typical data obtained in the initial velocity studies with 110,000 x G supernatants from V-79A and KC62 cells. Figure 1 data was obtained by varying ATP concentration at a constant, saturating (500 μM) concentration of R-5-P. Figure 2 data was obtained by varying R-5-P concentration with a constant, saturating (500 μM)

Figure 1. Double Reciprocal Plots of Initial Velocity Versus ATP Concentration.

PRPP-synthetase activity of 110,000 x G supernatant containing 40 μ g of total protein was assayed for 6 minutes at ATP concentrations ranging from 5 to 25 μ M at a constant R-5-P concentration of 500 μ M. Velocity is in nanomoles PRPP produced per minute. Kinetic parameters derived from the Wilkinson analysis were used to calculate the lines shown. Part A is for the V-79A enzyme. Kinetic parameters determined from this trial were K_m 9.57 ± 1.85 μ M and V_{max} 3.24 ± 0.24 nanomoles PRPP/min/mg of protein. Part B is for the KC62 enzyme. Kinetic parameters determined from this trial were K_m 8.40 ± 2.44 μ M and V_{max} 2.92 ± 0.30 nanomoles PRPP/min/mg of protein.

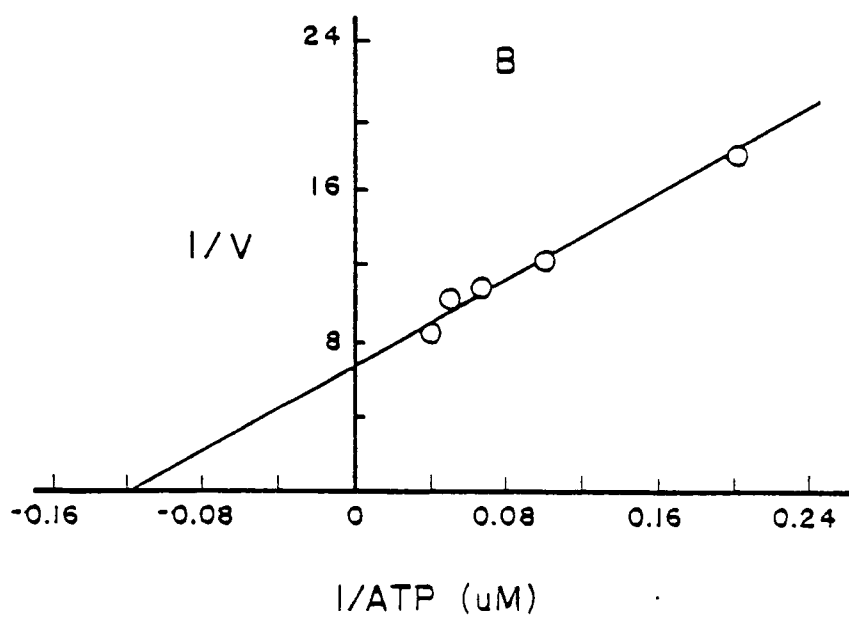
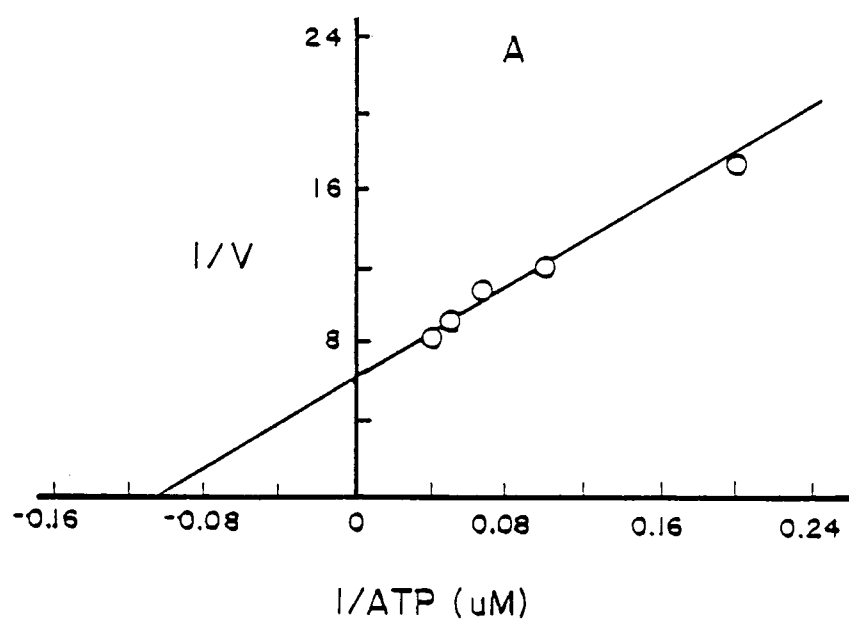


Figure 1.

Figure 2. Double Reciprocal Plots of Initial Velocity Versus R-5-P Concentration.

PRPP-synthetase activity of 110,000 x G supernatant containing 40 μ g of total protein was assayed for 6 minutes at R-5-P concentrations ranging from 5 to 40 μ M at a constant ATP concentration of 500 μ M. Velocity is in nanomoles PRPP produced per minute. Kinetic parameters derived from the Wilkinson analysis were used to calculate the lines shown. Part A is for the V-79A enzyme. Kinetic parameters determined from this trial were K_m 23.5 $\pm$ 2.70 μ M and V_{max} 3.64 $\pm$ 0.26 nanomoles PRPP/min/mg of protein. Part B is for the KC62 enzyme. Kinetic parameters determined from this trial were K_m 23.9 $\pm$ 2.57 μ M and V_{max} 3.68 $\pm$ 0.20 nanomoles PRPP/min/mg of protein.

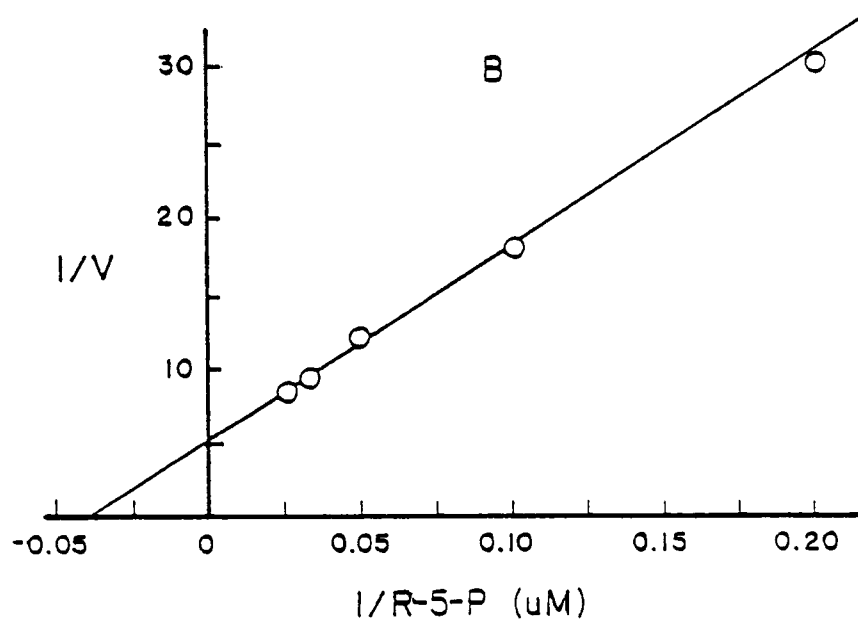
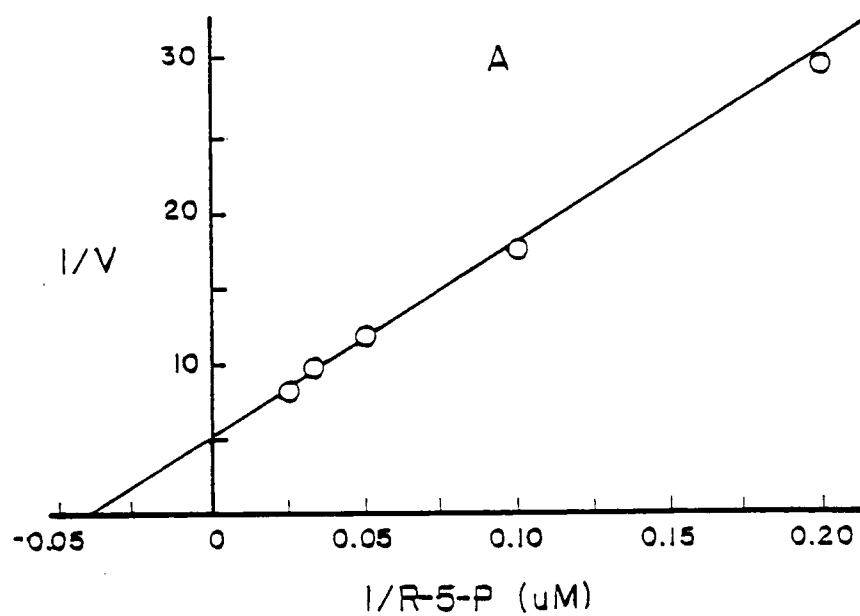


Figure 2.

concentration of ATP. Part A of each figure is the V-79A enzyme and Part B is the KC62 enzyme. Figures 1 and 2 show the actual experimentally obtained data points. While the results are displayed in a double reciprocal form, the best line (shown) and the apparent K_m and V_{max} were actually determined by the method of Wilkinson (33).

Table I shows the apparent K_m and V_{max} values for the V-79A and KC62 PRPP-synthetases. Table I values represent average values resulting from two separate trials on two different 110,000 x G supernatant preparations from each cell type.

Discussion

From the results shown in Table I, it appeared that no significant differences existed in the K_m and V_{max} values of the PRPP-synthetases of V-79A and KC62. This finding suggested that reduced intracellular PRPP levels in KC62 cells could not be attributed to an increased K_m value or decreased V_{max} value for KC62's PRPP-synthetase.

Similar kinetic studies have been done by Wong and Murray (24) with PRPP-synthetase from Ehrlich ascites tumor cells and by Green and Martin (26) with PRPP-synthetases from two, cultured rat hepatoma cell lines. In both studies, crude preparations of PRPP-synthetase were used and the K_m values were determined using pseudo-first order reaction conditions. PRPP-synthetase from Ehrlich ascites cells had apparent K_m values for ATP and R-5-P of 60 μM and 50 μM , respectively. PRPP-synthetases from both the rat hepatoma cell lines had K_m values for ATP and R-5-P of 45 μM and 200 μM , respectively. The K_m values determined in both these studies are substantially larger than those for the enzyme from V-79A or KC62.

TABLE I.
AVERAGE APPARENT KINETIC PARAMETERS FOR 110,000 X G
SUPERNATANT PRPP-SYNTHESES

Variable Substrate	V-79A		KC62	
	K_m^a	V_{max}^b	K_m^a	V_{max}^b
ATP	8.25 ± 0.15	3.20 ± 0.28	7.64 ± 0.67	3.36 ± 0.44
R-5-P	22.90 ± 2.41	3.66 ± 0.26	20.6 ± 2.15	3.52 ± 0.28

^a K_m determinations were done twice. Values represent the mean $\pm$ 1 S.D. K_m has the units μ moles/liter.

^b V_{max} determinations were done twice. Values represent the mean $\pm$ 1 S.D. V_{max} has the value nanomoles PRPP produced/min/mg of protein.

C. Inhibitor Studies

Materials and Methods

PRPP-synthetase activity was determined by using the same coupled enzyme assay as described in Materials and Methods for the Initial Velocity Studies. The final assay mixture contained 50 mM Na_3PO_4 (pH 7.4), 6 mM MgCl_2 , 500 μM ATP, 500 μM R-5-P, 16.4 μM [carboxy- ^{14}C]orotic acid, 0.5 mg OPRT-ODC enzyme and 100 μl of diluted 110,000 x G supernatant. Also included were sodium salts of mono-, di-, and triphosphate nucleotides which were purchased from the Sigma Chemical Company. The final assay volume was 500 μl .

A 20 ml glass scintillation vial containing all assay components except the 110,000 x G supernatant was warmed for 6 minutes at 37°C. A 100 μl volume of diluted 110,000 x G supernatant was pipetted into the vial, and the vial was immediately capped. The cap was lined with a 2 cm disk of Whatman 3 MM paper which had been wetted with 70 μl of 1.0 M KOH. The capped vial was incubated for 15 minutes at 37°C at which time the paper disk was removed from the cap, terminating collection of $^{14}\text{CO}_2$. Radioactivity of the disk was determined by scintillation counting. Nanomoles of PRPP generated was calculated using standard curves generated in a manner previously described.

Results

Table II shows the results of three different experiments in which KC62 and V-79A PRPP-synthetase activity was determined in the presence of a variety of mono-, di-, and triphosphate nucleotide inhibitors. Each experiment was done with a different pair of V-79A and KC62 110,000 x G supernatants. In all three experiments the activity of the V-79A and KC62 enzymes was

very similar in the absence of nucleotide inhibitors. In Experiments 1 and 2, two different concentrations of each AMP and ADP were tested. Under all inhibitory conditions tested in both experiments, the resulting degree of inhibition was greater for the PRPP-synthetase activity of KC62 than for its V-79A counterpart. The phenomenon was particularly apparent in the case of 0.1 mM AMP where activity of the KC62 enzyme was approximately half that of the V-79A enzyme. In Experiment 3, PRPP-synthetase activity was tested in the individual presence of seven other nucleotides. In all but two cases, 0.1 mM GDP and 1.0 mM GDP, inhibition of PRPP-synthetase activity was observed to be greater for the KC62 enzyme than for the V-79A enzyme. The most striking differences in inhibition were in the cases of 0.1 mM ITP and 0.1 mM XMP where the resulting activity of the V-79A enzyme was at least twice that of the KC62 enzyme.

Discussion

In all three experiments the activities of V-79A and KC62 PRPP-synthetase were similar in the absence of nucleotide inhibitors. This finding confirmed the results of the Initial Velocity Studies which demonstrated no significant differences in the catalytic activity of the enzyme from the two cell lines. The inhibitor studies demonstrated that the PRPP-synthetases from both cell lines were subject to inhibition by a variety of purine and pyrimidine nucleotides. This finding was expected since PRPP-synthetases from a wide variety of sources have previously been shown to be subject to nucleotide inhibition (20,24,25,26,34,35). Unexpected was the finding that XMP caused significant inhibition, since PRPP-synthetases from other sources tested have shown little inhibition by this nucleotide (23,35). The results of the inhibitor

TABLE II.

INHIBITION OF PRPP-SYNTHETASE ACTIVITY BY NUCLEOTIDES

Nucleotide Inhibitor	PRPP-Synthetase Activity ^a (nanomoles PRPP/mg protein/15 min)			
	V79A		KC62	
<u>Experiment 1</u>				
None	61	(100)	57	(100)
0.1 mM AMP	59	(97)	29	(51)
1.0 mM AMP	23	(38)	16	(28)
0.1 mM ADP	60	(98)	45	(79)
1.0 mM ADP	37	(61)	30	(53)
<u>Experiment 2</u>				
None	54	(100)	50	(100)
0.1 mM AMP	49	(91)	26	(52)
1.0 mM AMP	13	(24)	10	(20)
0.1 mM ADP	52	(98)	39	(78)
1.0 mM ADP	27	(50)	18	(36)
<u>Experiment 3</u>				
None	57	(100)	52	(100)
0.1 mM IMP	67	(118)	52	(100)
0.5 mM IMP	84	(147)	42	(81)
1.0 mM IMP	84	(147)	43	(83)
0.1 mM IDP	57	(100)	45	(86)
0.5 mM IDP	57	(100)	38	(73)
1.0 mM IDP	43	(75)	26	(50)
0.1 mM ITP	58	(102)	23	(44)
0.5 mM ITP	58	(102)	37	(71)
1.0 mM ITP	57	(100)	35	(67)
0.1 mM GDP	47	(82)	48	(98)
0.5 mM GDP	49	(86)	40	(77)
1.0 mM GDP	33	(59)	42	(81)
0.1 mM GTP	58	(102)	52	(100)
0.5 mM GTP	53	(93)	42	(81)
1.0 mM GTP	52	(91)	32	(62)

TABLE II (continued)

Nucleotide Inhibitor	PRPP Synthetase Activity ^a (nanomoles PRPP/mg protein/15 min)			
	V79A		KC62	
0.1 mM TMP	85	(149)	35	(67)
0.5 mM TMP	72	(126)	32	(62)
1.0 mM TMP	58	(102)	31	(60)
0.1 mM XMP	53	(93)	21	(40)
0.5 mM XMP	27	(47)	12	(23)
1.0 mM XMP	18	(32)	7	(13)

^aValues in parentheses are the percent of activity relative to activity with no inhibitors. Different cell extracts were used for each experiment.

studies further support the idea that nucleotide inhibition is an important regulatory mechanism under physiological conditions. From Table II it can be seen that the PRPP-synthetases from both cell lines underwent a significant degree of inhibition at nucleotide concentrations comparable to those found in cultured cells. More importantly, however, the results of these inhibitor studies suggest that the KC62 PRPP-synthetase had an increased sensitivity to a variety of nucleotide inhibitors when compared to its V-79A counterpart.

It has previously been shown that KC62's steady state PRPP levels are approximately 30% of those of V-79A (27). It is reasonable to believe that KC62's reduced PRPP levels are a direct consequence of altered regulatory properties of its PRPP-synthetase. Since the major intracellular nucleotide pool sizes of KC62 and V-79A are the same (See Chapter V, High Pressure Liquid Chromatography, Results), it would be expected that the degree of inhibition for their respective PRPP-synthetases would be equivalent. However, increased sensitivity to nucleotide inhibition on the part of KC62's PRPP-synthetase would result in a greater decrease in its activity than its V-79A counterpart. Decreased KC62 PRPP-synthetase activity would lead to decreased PRPP production. Assuming the rate of PRPP utilization by V-79A and KC62 cells is similar it would be reasonable to think that the steady state level of PRPP might be less in KC62 cells.

Numerous investigators have examined the relationship between PRPP-synthetase activity and PRPP levels. Several investigators have demonstrated that increased availability of R-5-P, in vivo, may result in increased PRPP-synthetase activity and expansion of PRPP levels (21,22,23). Other investigators have discovered mutant forms of PRPP-synthetase and have investigated the

consequences on PRPP levels. Valentine et al. (36,37,38) have investigated PRPP-synthetase activity of dialyzed hemolysates of erythrocytes from an individual suffering from nonspherocytic hemolytic anemia. PRPP-synthetase activity was found to be approximately 40 to 60% of normal and accompanied by a 50% reduction in PRPP levels. Wada et al. (39), using a similar erythrocyte preparation from a hypouricemic infant, found PRPP-synthetase activity to be only 10% of normal and accompanied by a 50% reduction in PRPP levels. More common have been reports of mutant forms of PRPP-synthetase with hyperactivity and accompanied by increased PRPP levels. Three distinctly different mutant forms of hyperactive PRPP-synthetase have been demonstrated in cultured fibroblasts from individuals suffering from hyperuricemia and gout. Becker et al. (20,40,41,42,43) have reported such a fibroblastic PRPP-synthetase with a V_{max} 2.5 to 3.0 fold greater than normal. In these fibroblasts PRPP-synthetase activity was 3 to 4 fold greater than normal and accompanied by PRPP levels twice normal. Becker et al. (44) have also reported a second distinct mutant form of PRPP-synthetase in cultured fibroblasts. This mutant PRPP-synthetase had a greatly decreased K_m for R-5-P, resulting in an increased PRPP-synthetase activity in vivo and expanded PRPP levels. A third mutant form of fibroblastic PRPP-synthetase has been reported by Sperling et al. (45,46). The mutant enzyme had normal catalytic properties, but exhibited a decreased sensitivity to inhibition by nucleotides. The PRPP-synthetase was inhibited by GDP, ADP and AMP, but under the assay conditions employed an average of 45, 5, and 1.5 fold higher concentrations, respectively, were required to inhibit the mutant enzyme to the same extent as the normal enzyme. As in previous reports, the cells with the mutant enzyme exhibited increased PRPP-synthetase activity in vivo and increased PRPP levels.

The findings of these investigators are highly supportive of the idea that KC62's decreased PRPP levels can be attributed to decreased PRPP-synthetase activity in vivo. They also demonstrate that distinctly different mutant forms of PRPP-synthetase are possible, thus, lending support to the idea that KC62's PRPP-synthetase could be structurally altered in such a manner as to exhibit increased sensitivity to inhibition by nucleotides.

CHAPTER IV

PURIFIED PRPP-SYNTHETASE FROM V-79A AND KC62

A. Purification of PRPP-Synthetase

Materials and Methods

In all steps of the purification PRPP-synthetase activity was determined by the procedure outlined in Chapter III, Initial Velocity Studies, Materials and Methods. Assay duration was 6 minutes, and ATP and R-5-P concentration were each 500 μ M. Protein was determined by the method of Lowry (30), using bovine serum albumin as a standard.

The medium was removed from six roller bottles of 80% confluent cells and the cells were washed twice with 37°C Hanks' balanced salt solution (20 ml/roller bottle). The cells were trypsinized and resuspended in a total of 125 ml of 37°C LAC medium. The cells were collected by centrifugation at 120 x G for 5 minutes. The resultant cell pellets were washed once with a total of 120 ml of 37°C Hanks' balanced salt solution and repelleted by centrifugation. The cell pellets were resuspended in 36 ml of 4°C Extraction Buffer consisting of 50 mM Na_3PO_4 (pH 7.0), 6 mM MgCl_2 , 60 μ M ATP and 0.1 mM DTT. The cells were lysed by freezing in liquid nitrogen and thawing five times. The lysate was centrifuged at 30,000 x G for 30 minutes at 4°C.

DEAE-cellulose (Whatman DE-52) was prepared according to the manufacturer's specifications as outlined in Whatman publication IL2. The DEAE-cellulose was poured into a column (1.5 x 6.0 cm) and had a final volume of 10 ml. The resin was equilibrated at 4°C with Extraction Buffer. The 30,000 x G supernatant of the cell lysate was applied to the column and washed on 25 ml of Extraction Buffer. During the loading and washing on of the

30,000 x G supernatant, 26 fractions of 2.2 ml each were collected. A linear gradient was then started with 25 ml of Extraction Buffer in one chamber of a gradient maker (Hoefer Scientific Instruments) and 25 ml of Extraction Buffer containing 300 mM KCl in the other chamber. Twenty-three fractions of 2.2 ml each were collected. PRPP-synthetase activity was eluted off the column as a single peak in Fractions 30-42. The four or five fractions having the highest specific activity were pooled and concentrated to approximately 0.8 ml in a 3 ml ultrafiltration cell (Millipore Corporation) using a Millipore PSED filter (M.W.C.O. 10,000).

The concentrated PRPP-synthetase peak from the DEAE-cellulose column was applied to an 18 ml (0.9 x 2.8 cm) Sepharose 6B (Pharmacia Fine Chemicals) column which had been pre-equilibrated at 4°C with a Sepharose Buffer containing 50 mM Na_3PO_4 (pH 7.4), 6 mM MgCl_2 , 0.3 mM ATP and 0.1 mM DTT. The column was eluted with Sepharose Buffer and 30 fractions of 0.54 ml each were collected. The PRPP-synthetase activity eluted off the column as a single peak in Fractions 10-20.

Powdered, high resolution hydroxylapatite (HAP) (Calbiochem-Behring Corporation) was hydrated with 50 mM Na_3PO_4 buffer (pH 7.4) containing 6 mM MgCl_2 , 0.3 mM ATP and 0.1 mM DTT. The HAP suspension was poured into a 0.45 x 3.5 cm column and had a final volume of 0.5 ml. The resin was equilibrated with Hydration Buffer at 4°C.

The four or five fractions from the Sepharose 6B column having the highest specific activity were pooled and applied to the HAP column. A step gradient of 70 mM, 150 mM, 170 mM and 400 mM KCl Hydration Buffer (pH 7.4) was used to elute the column. Fractions of 0.27 ml each were collected. PRPP-synthetase activity eluted from the column as a single peak in the 150

mM step. The three or four fractions containing the highest specific activity were pooled, placed in a Spectrapor 2 membrane, and dialyzed against 500 volumes of 50 mM Na_3PO_4 Hydration Buffer (pH 7.4) for 4 hours. Aliquots of the dialyzed enzyme were stored frozen at -70°C .

Prior to its use in the initial velocity and inhibitor studies the enzyme was desalted to remove ATP. Desalting of the thawed enzyme was performed on 0.5 ml or 1.0 ml Sephadex G-25 (Pharmacia Fine Chemicals) columns pre-equilibrated with 50 mM Na_3PO_4 buffer (pH 7.4) containing 6 mM MgCl_2 and 0.1 mM DTT. The enzyme was eluted from the column with the same buffer as used for column pre-equilibration.

Results

Figures 3, 4 and 5 show typical elution patterns of V-79A PRPP-synthetase activity from the DEAE, Sepharose 6B and HAP columns, respectively. It can be seen in Figure 3 that a large amount of the applied 30,000 x G supernatant protein is not retained on the column and passes straight through the column during the loading and washing procedures (Fractions 1-26). The PRPP-synthetase activity eluted as a single peak shortly after the start of the KCl gradient. It can be seen in Figure 4 that the V-79A PRPP-synthetase activity eluted from the Sepharose 6B column as a single peak prior to elution of the majority of applied protein. It can be seen in Figure 5 that the V-79A PRPP-synthetase activity eluted from the HAP column as a single peak at the 150 mM KCl step of the gradient. A large protein peak coincides with the V-79A PRPP-synthetase activity peak.

The elution profiles of KC62 PRPP-synthetase activity from each of the three columns during purification was found to be identical to that of V-79A

Figure 3. DEAE-Cellulose Chromatography Elution Profile of V-79A PRPP-Synthetase.

Each fraction contained 2.2 ml. Protein concentration (○) is in mg/ml. PRPP-synthetase activity (Δ) is given as the CPM produced for a standard 6 minute assay with ATP and R-5-P each at 500 μ M. The KCl concentration (—) of the elution buffer is in terms of millimolar.

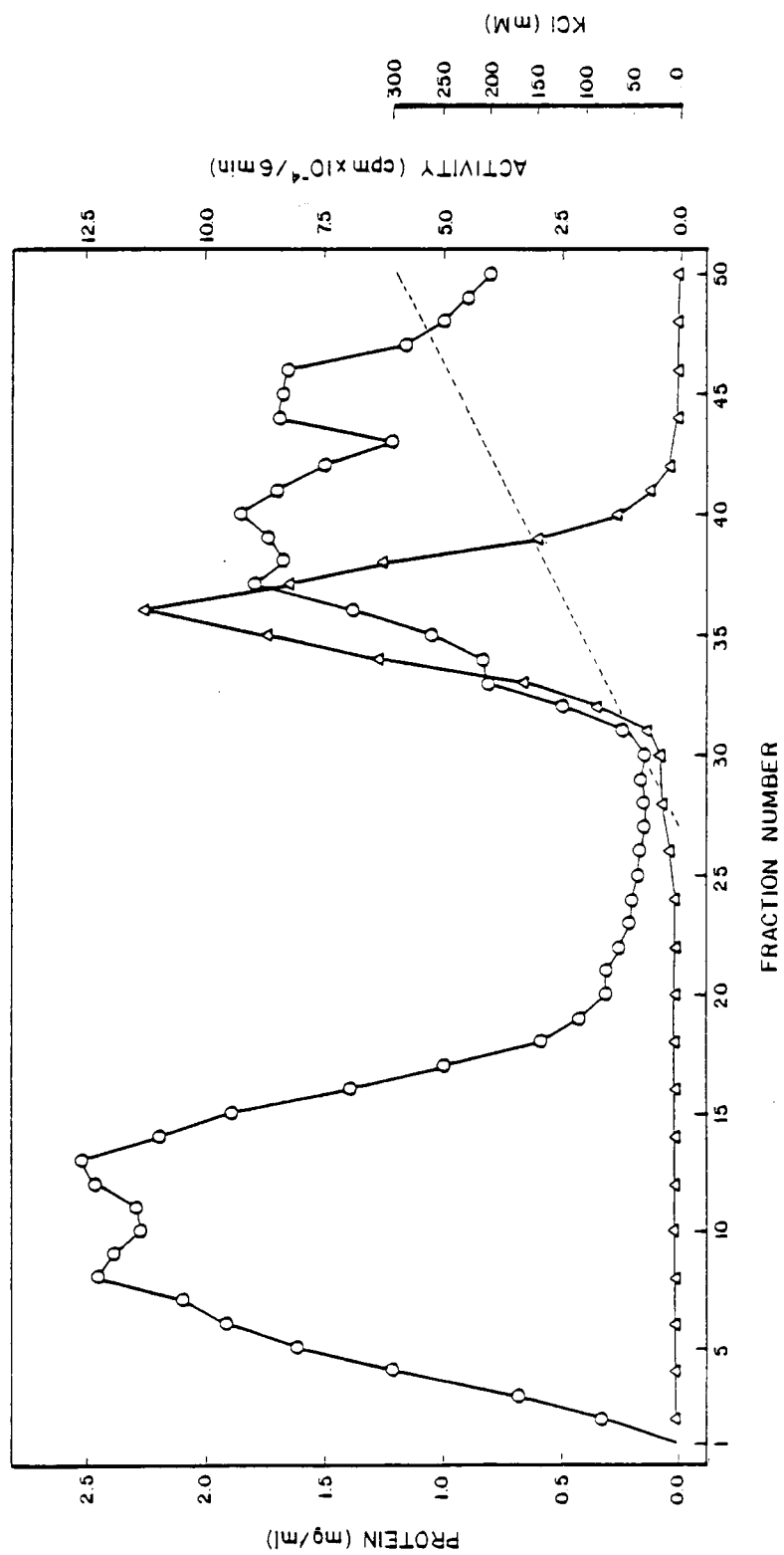


Figure 3.

Figure 4. Sepharose 6B Chromatography Elution Profile of V-79A PRPP-Synthetase.

Each fraction contained 0.54 ml. Protein concentration (O) is in mg/ml. PRPP-synthetase activity (Δ) is given as CPM produced for a standard 6 minute assay with ATP and R-5-P each at 500 μ M.

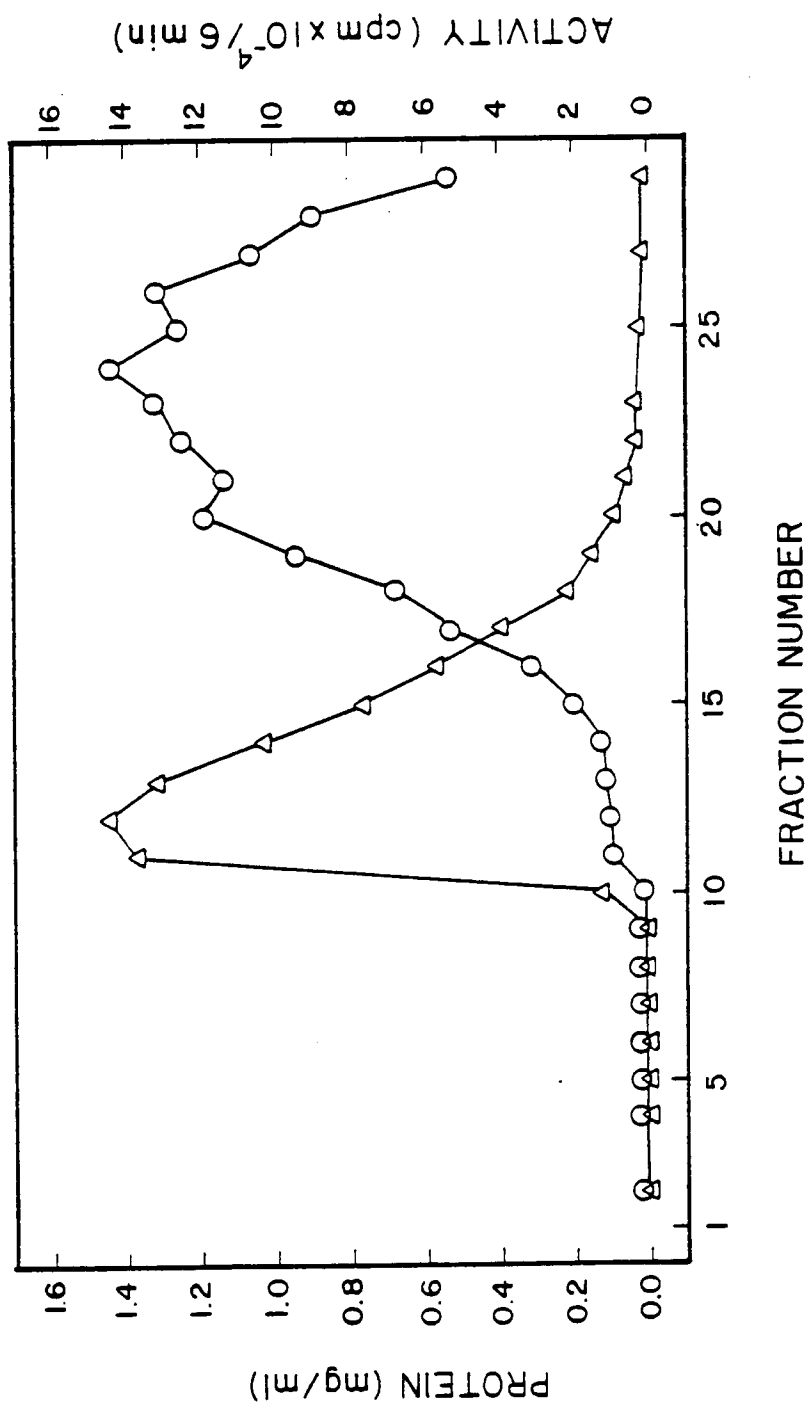


Figure 4.

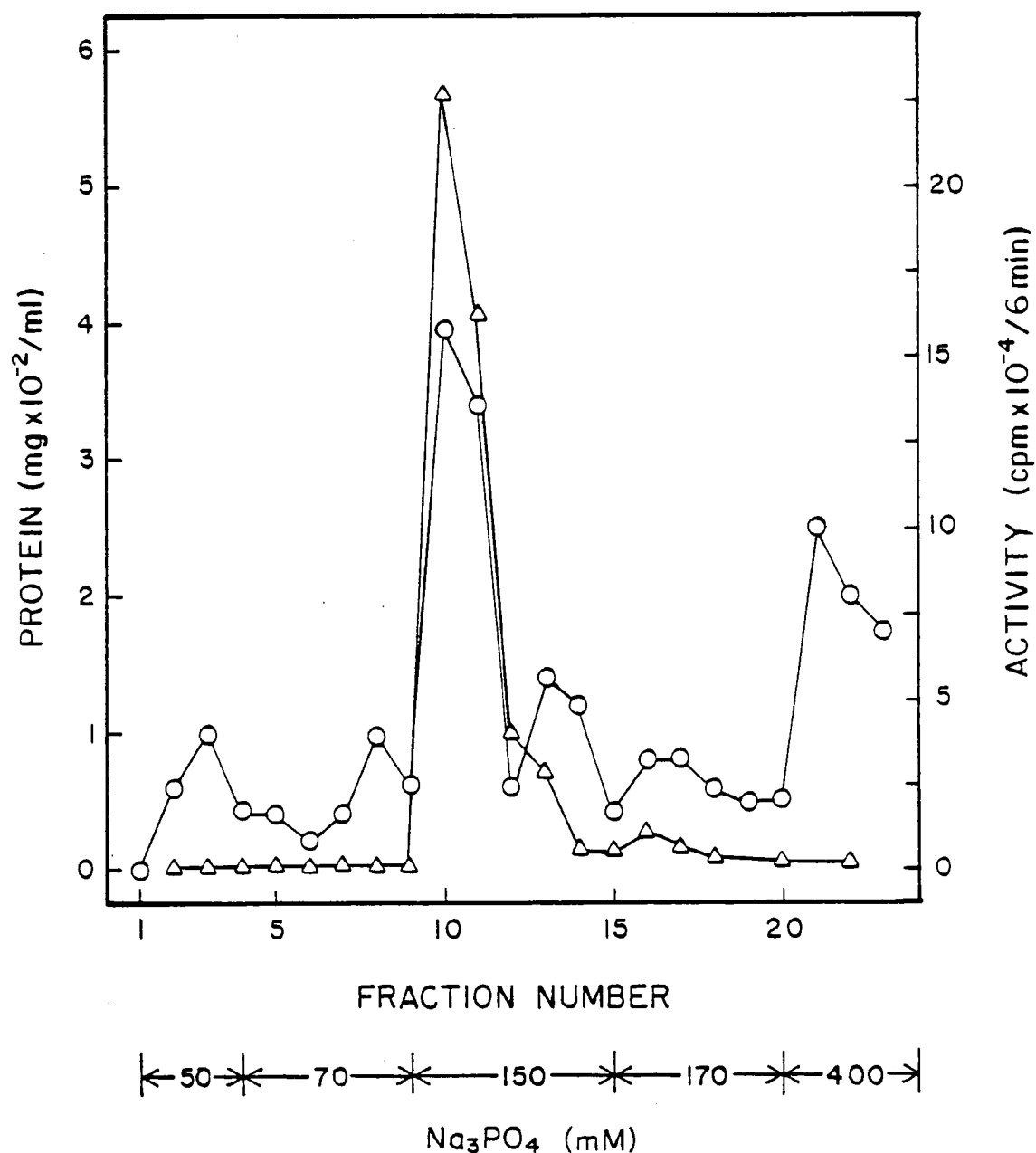


Figure 5. Hydroxylapatite Chromatography Elution Profile of V-79A PRPP-Synthetase.

Each fraction contained 0.27 ml. Protein concentration (O) is given in mg/ml. PRPP-synthetase activity (Δ) is given as CPM produced for a standard 6 minute assay with ATP and R-5-P each at 500 μ M. KCl concentration of the elution buffers are given in terms of millimolar.

PRPP-synthetase activity. This suggested that no significant physical difference existed between the V-79A and KC62 PRPP-synthetases.

A representative example of V-79A PRPP-synthetase purification results is shown in Table III. The purification procedure was carried out several different times with a final recovery as high as 13% and purification up to 1000 fold. Similar final recovery and fold purification was obtained for the KC62 enzyme.

Discussion

Purification schemes for PRPP-synthetases from a number of sources have been developed by several investigators. Such schemes have generally employed large quantities of starting material and resulted in relatively poor final recovery of activity. These schemes were judged to be impractical for purification of PRPP-synthetase from V-79A or KC62 cells because of the limited quantities of starting material available. It was therefore necessary to develop a purification scheme that could be used with small quantities of starting material and provide a reasonable final recovery of activity.

A variety of purification techniques were tested singularly and in combination to assess resulting fold purification and percent recovery of activity. Techniques tested included some previously employed by other investigators as well as others (e.g. HAP) which had not previously been used with this enzyme. The results of these preliminary experiments allowed development of the purification scheme subsequently employed. While not providing complete purification, significant purification and reasonable recovery were achieved utilizing only a small amount of starting material.

PRPP-synthetases from several sources have been purified by other investigators. Switzer (18), using a six step procedure, has purified the

TABLE III.
PURIFICATION OF PRPP-SYNTHETASE

Step	Total Protein (mg)	Specific Activity (Units ^a /mg)	Purification (fold)	Total Enzyme (Units) ^a	Recovery (%)
1. Lysate	225.00	4.02	1.0	904.5	100.0
2. Supernatant	136.37	6.44	1.6	878.2	97.1
3. DEAE-Cellulose	15.04	41.38	10.3	622.4	68.8
4. Sepharose 6B	0.26	826.88	305.7	215.0	23.8
5. Hydroxylapatite	0.02	3744.38	932.4	74.9	8.3

^aOne unit of enzymatic activity is defined as that amount of enzyme which produces one nanomole of PRPP/minute at 37°C.

Salmonella typhimurium enzyme 450 fold with a 25% recovery of activity. Roth et al. (19), using a five step procedure, have purified the rat liver enzyme 1,020 fold with a 9.8% recovery of activity. Fox and Kelly (17), using a six step procedure, have purified the human erythrocyte enzyme 5,100 fold with a 6.4% recovery of activity.

B. Physical Properties of PRPP-Synthetase

Materials and Methods

Sodium dodecyl sulfate electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (47) using a vertical slab electrophoresis cell (Hoefer Scientific Instruments). The Tris-HCl (Fisher Scientific Company) buffered (pH 6.8) stacking gel contained 3% polyacrylamide (Bio-Rad Laboratories), 0.1 % SDS (Bio-Rad Laboratories), and was approximately 1.5 cm in length. The Tris-HCl buffered (pH 8.8) separating gel contained 10% polyacrylamide, 0.1% SDS and was approximately 12.5 cm in length. The electrode buffer (pH 8.8) contained 0.32 M glycine (Bio-Rad Laboratories), 0.042 M Tris-HCl, and 0.1% SDS. Dried protein samples were dissolved in a sample buffer containing 0.062 M Tris-HCl, 20% glycerol (Fisher Scientific Company), 1.0% SDS, 10% 2-mercaptoethanol (Sigma Chemical Company) and 0.05% bromophenol blue (Matheson Company, Inc.), and incubated for 10 minutes in a boiling water bath prior to their use. Molecular weight standards used included phosphorylase B (M.W. = 92,500), bovine serum albumin (M.W. = 67,000), ovalbumin (M.W. = 43,000), and myoglobin (M.W. = 17,000). All molecular weight standards were products of the Sigma Chemical Company. Protein samples were

electrophoresed at a constant current of 6mA during stacking and 17mA during separation. Gels were silver stained by the method of Morrissey (48).

Molecular weight determination by gel filtration. Molecular weight determination was carried out with a Sepharose 6B column and an elution buffer containing 50 mM Na₃PO₄ (pH 7.4), 6mM MgCl₂, 0.3mM ATP, and 0.1 mM DTT. The Sepharose 6B column was 48.5 cm in length and 1.0 cm in diameter, and 0.49 ml fractions were collected. The column was calibrated with Blue Dextran 2000 (1 mg/ml), aldolase (5 mg/ml), catalase (5 mg/ml), ferritin (1 mg/ml), and thyroglobulin (5 mg/ml). All standards used for column calibration were purchased from Pharmacia Fine Chemicals. A 0.4 ml volume of each protein standard was individually chromatographed to determine elution volume. Elution of the standards from the column was determined by monitoring absorbance at 280 nm. A 0.4 ml volume containing 50 µg of purified PRPP-synthetase having a specific activity of 1,230 units/mg (300 fold purification) was used to determine PRPP-synthetase elution volume. Elution of the enzyme from the column was determined by monitoring enzymatic activity. Approximately 30 µl of each fraction was assayed for activity by the procedure outlined in Chapter III, Initial Velocity Studies, Materials and Methods. The K_{av} value of each protein was calculated using the equation

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume of the protein, V_o = column void volume, and V_t = total bed volume.

Thermal inactivation of PRPP-synthetase. Thermal inactivation of purified PRPP-synthetases from V-79A and KC62 cells were studied at 55°C. Fifty microliter aliquots containing 0.5 µg of PRPP-synthetase having a specific

activity of 2,050 units/mg (500 fold purification) in 50mM Na_3PO_4 buffer (pH 7.4) containing 6mM MgCl_2 and 0.3mM ATP were added to glass test tubes (10 x 75 mm) sitting in a 55°C water bath. The tubes were incubated for 0.5 to 5.5 minutes at which time they were immediately placed in an ice-water bath. A 25 μl aliquot of the heat-treated enzyme was removed from each test tube and assayed for activity according to the procedure outlined in Chapter III, Initial Velocity Studies, Materials and Methods. The activity assay was 6 minutes in duration and ATP and R-5-P concentration were each 500 μM .

Results

Sodium dodecyl sulfate electrophoresis. Figure 6 shows the SDS electrophoresis gel of purified (900 fold) PRPP-synthetases from V-79A (Lane II) and KC62 (Lane III). For both PRPP-synthetase preparations a single major band of identical mobility is present. This is presumed to represent the PRPP-synthetase subunit. The identical mobility of this major band in both enzyme preparations suggests that no difference exists in the subunit molecular weight of the PRPP-synthetases from V-79A and KC62. Two other distinct minor bands of identical and lesser mobility are also present in the two enzyme preparations as well as several other faint bands. Figure 7 is a plot of the log of subunit molecular weight versus mobility of the protein standards from the gel shown in Figure 6. The major band present in both the V-79A and KC62 PRPP-synthetase preparations corresponds to a subunit molecular weight of about 27,000. The two other distinct minor bands seen in both enzyme preparations have subunit molecular weights corresponding to 135,000 and 74,000.

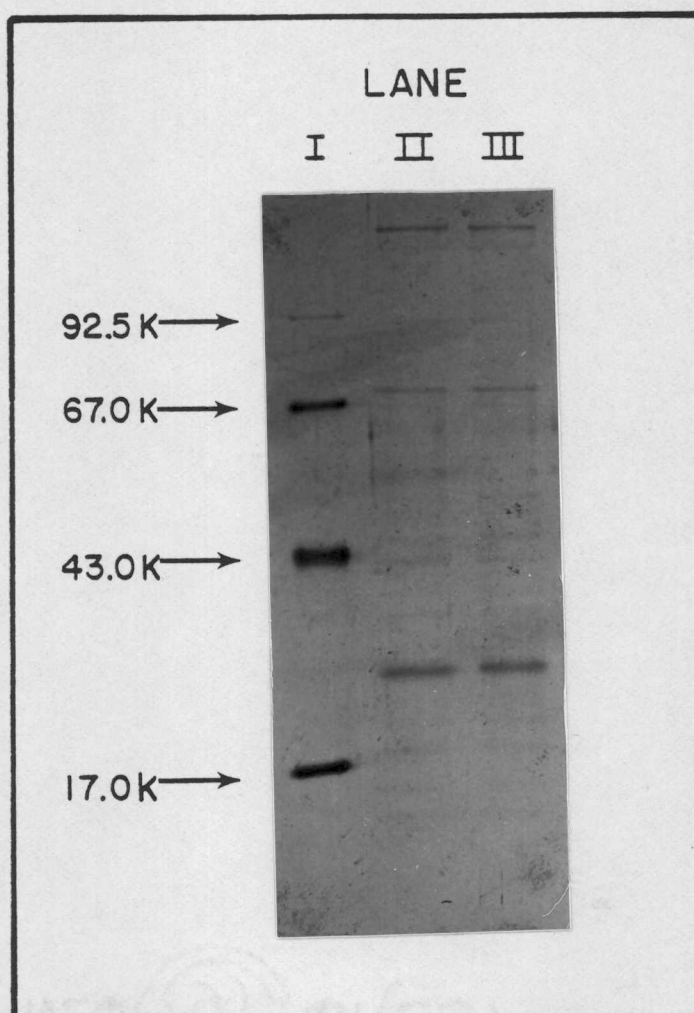


Figure 6. Sodium Dodecyl Sulfate Electrophoresis Gels of PRPP-Synthetase.

Lane I contains 2 μ g of phosphorylase B, 4 μ g of bovine serum albumin, 4 μ g of ovalbumin and 4 μ g of myoglobin. Lane II contains 1 μ g of purified (900 fold) V-79A PRPP-synthetase. Lane III contains 1 μ g of purified (900 fold) KC62 PRPP-synthetase.

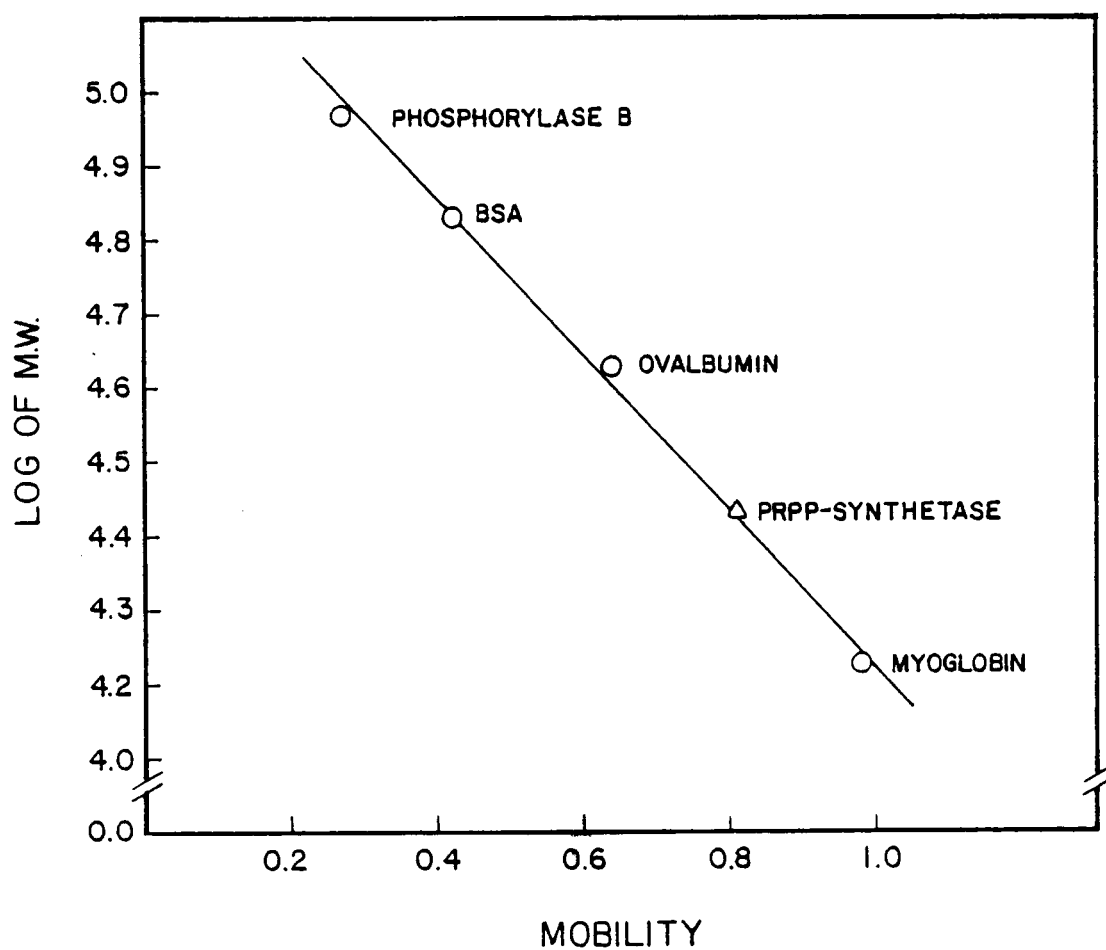


Figure 7. Subunit Molecular Weight Determination from Sodium Dodecyl Sulfate Electrophoresis Gels.

A plot of the log of molecular weight versus mobility demonstrates a subunit molecular weight of 27,000 for both V-79A and KC62 PRPP-synthetases.

Molecular weight determination by gel filtration. A plot of calculated K_{av} value versus log of molecular weight is shown in Figure 8. Both V-79A and KC62 PRPP-synthetase eluted from the column as a single, broad peak of activity and with identical elution volumes. The calculated K_{av} value for both PRPP-synthetases was 0.115 corresponding to a molecular weight of approximately 1,450,000. Assignment of this molecular weight assumes that the PRPP-synthetases are approximately globular and have partial specific volumes similar to those of the protein standards. The assigned molecular weight for the PRPP-synthetases probably contains a significant amount of error since PRPP-synthetase elution volume is approaching the void volume of the column.

Thermal inactivation of PRPP-synthetase. Thermal inactivation at 55°C of identical quantities of purified V-79A and KC62 PRPP-synthetase proceeded in an identical manner as shown in Figure 9. Approximately 90% of the initial activity is lost in four minutes at 55°C. A similar pattern of thermal inactivation at 60°C was observed for 110,000 X G preparations of the enzymes.

Discussion

Sodium dodecyl sulfate electrophoresis. At present it is unclear whether PRPP-synthetase from any tissue source has been purified to homogeneity. Roth et al. (19) found one major and three to five minor bands in SDS gels of a purified (3,000 fold) preparation of rat liver PRPP-synthetase. He concluded that the major band (90% of total stainable material) represented the PRPP-synthetase subunit and determined its molecular weight to be 40,500. Both Fox and Kelly (17), and Becker et al. (20) have attempted to determine the subunit molecular weight of human erythrocyte PRPP-synthetase. Fox, using a 5,100 fold purified enzyme preparation, determined the subunit molecular

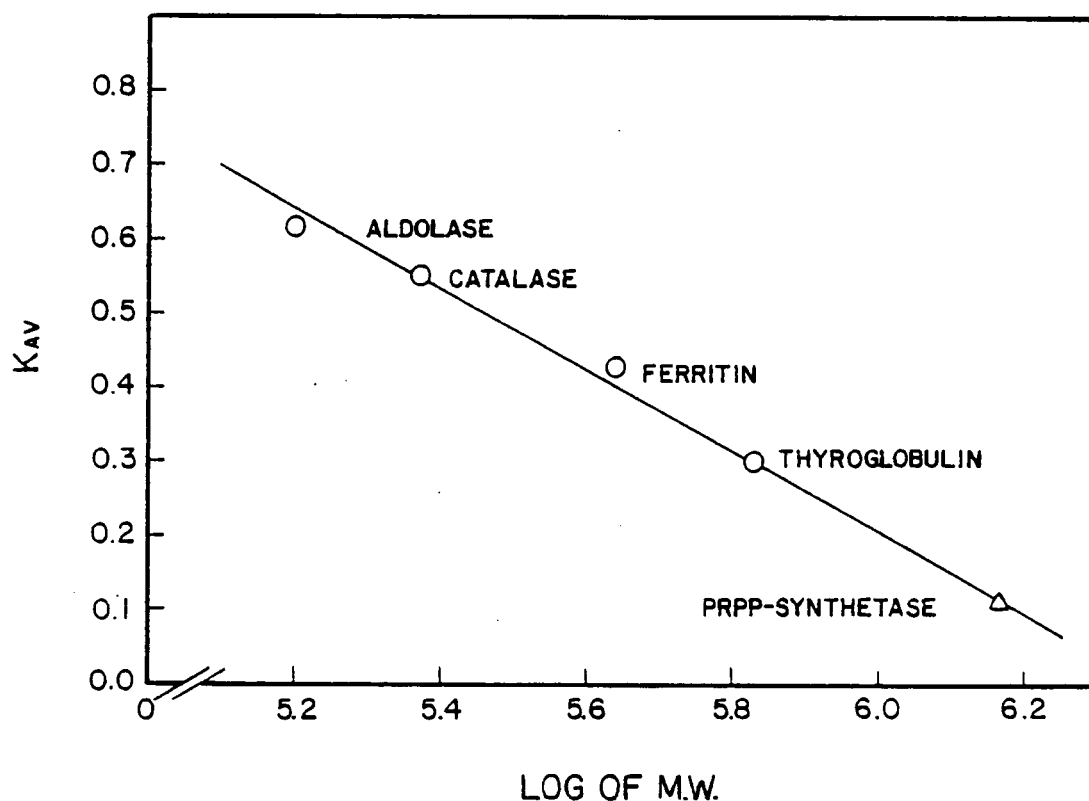


Figure 8. Molecular Weight Determination of PRPP-Synthetase from Sepharose 6B.

Protein standards were aldolase (M.W. = 158,000), catalase (M.W. = 232,000), ferritin (M.W. = 440,000) and thyroglobulin (M.W. = 669,000). The V-79A and KC62 PRPP-synthetases had identical elution volumes corresponding to a molecular weight of 1,450,000.

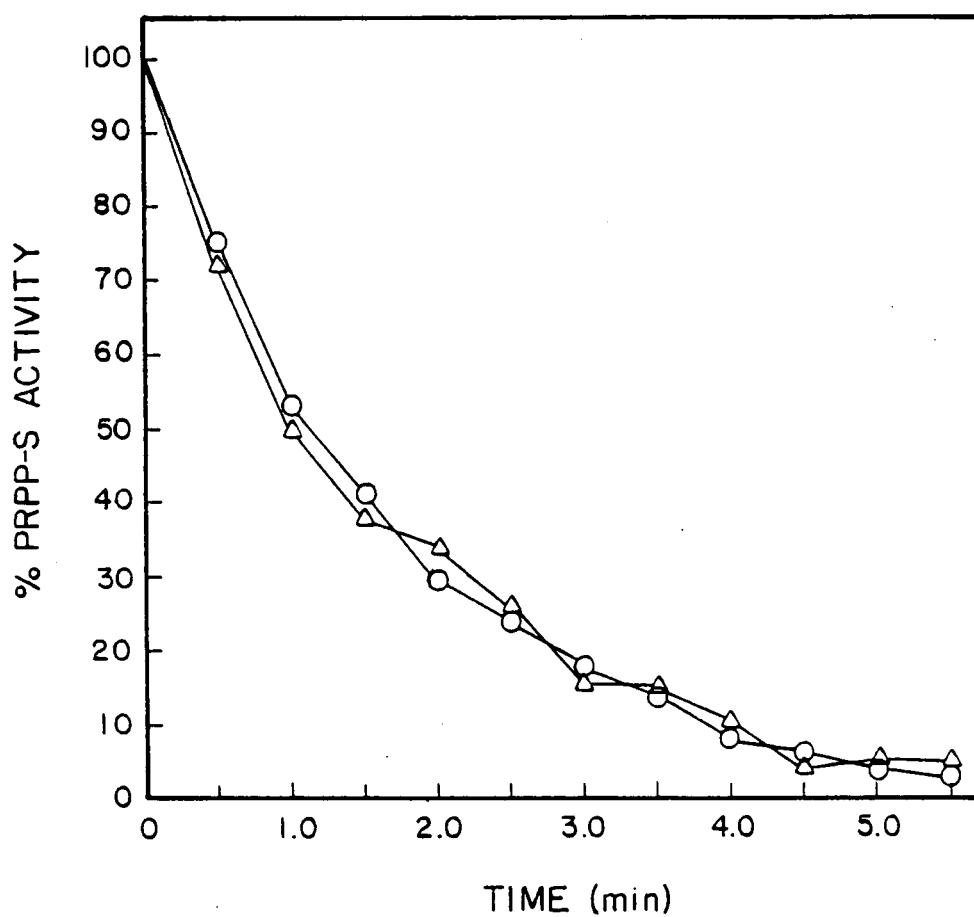


Figure 9. Thermal Inactivation of PRPP-Synthetase.

Purified V-79A and KC62 PRPP-synthetase were incubated at 55°C for 0.5 to 5.5 minutes and then assayed for activity. Percent activity remaining is the percentage of activity relative to the activity of the enzyme not incubated at 55°C. V-79A (O), KC62 (Δ).

weight to be 34,500. Becker, using a 4,500 fold purified enzyme preparation, determined the subunit molecular weight to be 32,000. While the subunit molecular weight values determined by Fox and Becker are similar, their studies fail to provide definitive proof that the enzyme consists of only a single type of subunit. In both studies SDS gels of the purified enzyme preparations contained more than a single band and identification of the PRPP-synthetase subunit band was made by inference.

The major band seen in SDS gels of purified PRPP-synthetase preparations from V-79A and KC62 cells is presumed to be the PRPP-synthetase subunit. Its molecular weight is about 27,000 which is similar to the subunit molecular weight values previously reported for PRPP-synthetases from other sources. The identical mobility of this major band in both enzyme preparations suggests that there is no difference in the subunit molecular weights of the V-79A and KC62 enzyme. Assuming that the V-79A and KC62 PRPP-synthetases have only a single type of subunit, it is unlikely that either of the two minor bands present on the gels represents the PRPP-synthetase subunit. Their estimated molecular weight values of 135,000 and 74,000 are significantly larger than any previously reported subunit molecular weight values for PRPP-synthetase.

Molecular weight determination by gel filtration. The large molecular weight values obtained for both V-79A and KC62 PRPP-synthetase suggest that in the presence of saturating ATP and $MgCl_2$ concentrations both enzymes exist as large multimeric proteins. Similar results have been obtained by other investigators for PRPP-synthetases from several sources. Fox and Kelly (17) have determined the molecular weight of the human erythrocyte enzyme by gel exclusion chromatography and sucrose density gradient ultracentrifugation. In the presence of saturating ATP and $MgCl_2$ concentrations the enzyme was

found to consistently aggregate into two "heavy forms" having molecular weights of 720,000 and 1,200,000. Smaller molecular weight forms were observed in the presence of ATP or $MgCl_2$ alone. Switzer (18) has determined the molecular weight of the enzyme from Salmonella typhimurium by sucrose density gradient ultracentrifugation. In the presence of saturating concentrations of $MgCl_2$ and no ATP, the enzyme was found to have a molecular weight of 540,000.

Thermal inactivation of PRPP-synthetase. The similar patterns of thermal inactivation of the V-79A and KC62 PRPP-synthetase suggests that no major structural difference exists between the two enzymes. Failure to demonstrate a difference in the pattern of thermal inactivation, subunit weight or molecular weight of the two enzymes is not unexpected. The clonal line KC62 was isolated from V-79A cells mutagenized with the alkylating agent ethylmethane sulfonate (EMS). EMS derives its primary mutagenic potential from its ability to induce point mutations (49,50). EMS induction of point mutations in the V-79 cell line has been demonstrated by Chu et al. (51,52). It seems likely, therefore, that EMS mutagenesis has resulted in only a "minor" structural alteration of the PRPP-synthetase of KC62 cells. This presumed structural alteration is apparently responsible for the enzyme's altered regulatory properties but has not significantly changed its catalytic or physical properties.

Becker et al. (20,42) have examined the thermal inactivation of a normal and mutant form of human erythrocyte PRPP-synthetase. The mutant enzyme had been shown to have a V_{max} 2.5 to 3.0 fold greater than the normal enzyme (20,40,44). With crude preparations of both enzymes, no difference in the pattern of thermal inactivation at 60°C was observed (42). With purified preparations, however, an increased rate of thermal inactivation at 55°C was observed for the mutant enzyme (20).

C. Initial Velocity Studies

Materials and Methods

The procedure by which the initial velocity studies of purified PRPP-synthetases were performed was identical to that outlined in Chapter III, Initial Velocity Studies, Materials and Methods, except for the manner in which substrate concentration was varied. In this case a series of fixed, subsaturating concentrations of the first substrate was used with each of a series of fixed, subsaturating concentrations of the second substrate. Approximately 0.5 μ g of protein having a specific activity of at least 2,050 units/mg (500 fold purification) was used for each determination. All assays were 6 minutes in duration. The resulting data was analyzed by a linear, least squares fit and apparent kinetic parameters were calculated.

Results

Double reciprocal plots of typical initial velocity data at variable ATP concentrations and changing, fixed concentrations of R-5-P are shown in Figure 10. Part A of Figure 10 is data for the V-79A enzyme and Part B is for the KC62 enzyme. All data for both enzymes plotted in a double reciprocal manner yielded a family of lines which intersect to the left of the vertical axis above the horizontal axis. Secondary plots of data from Figure 10 Part A and B are shown in Figures 11 and 12, respectively. In Figures 10, 11, and 12 the best lines were determined by a linear, least squares fit and the apparent kinetic parameters were calculated.

Table IV summarizes calculated, apparent K_m values for two trials for each enzyme type. Each trial was conducted with a different purified enzyme preparation.

Figure 10. Double Reciprocal Plots of Initial Velocity at Variable ATP Concentrations and Fixed R-5-P Concentrations.

The concentration of ATP was varied from 5 to 20 μM while R-5-P concentration was held constant at the values: 2 μM (∇), 3 μM ($\square$), 4 μM (Δ), 6 μM ($\diamond$), and 10 μM ($\circ$). Velocity is in nanomoles/min. Part A is for the V-79A enzyme. Part B is for the KC62 enzyme.

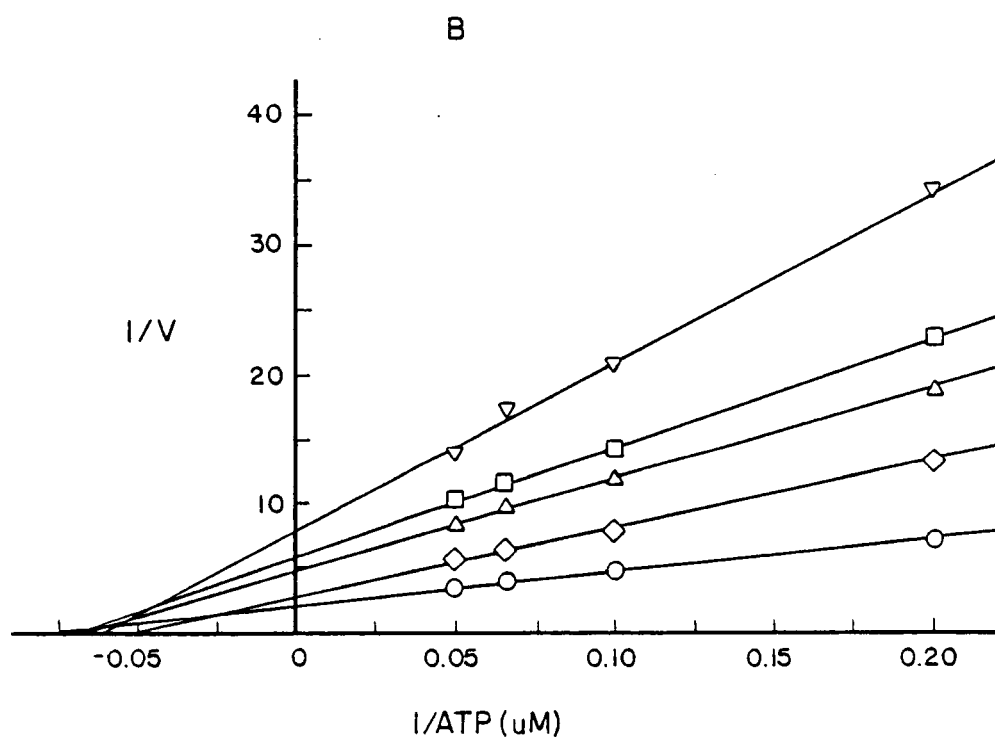
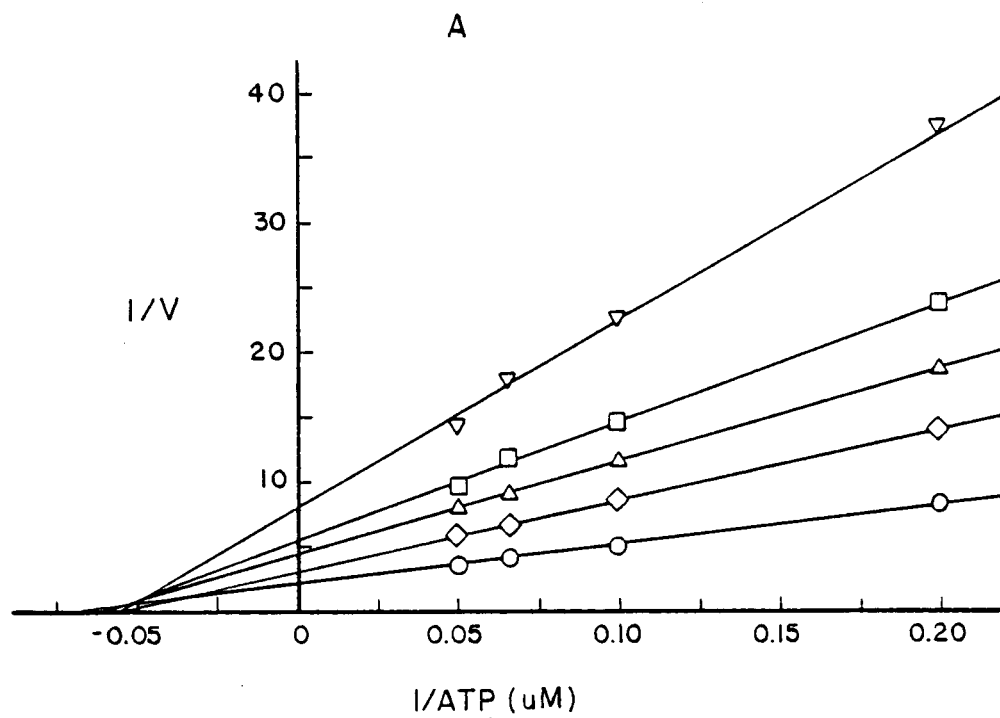


Figure 10.

Figure 11. Secondary Plots of Figure 10, Part A Results (V-79A).

The intercept plot (Part A) is the $1/V$ intercept values from Figure 10, Part A versus $1/R-5-P$ concentration. The slope plot (Part B) is the slope values from Figure 10, Part A versus $1/R-5-P$ concentration.

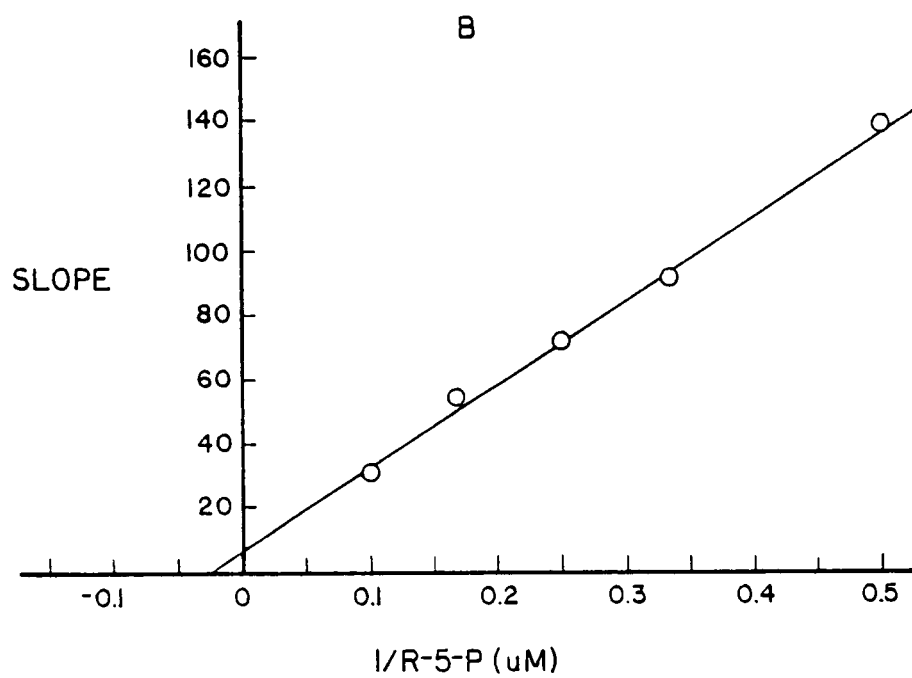
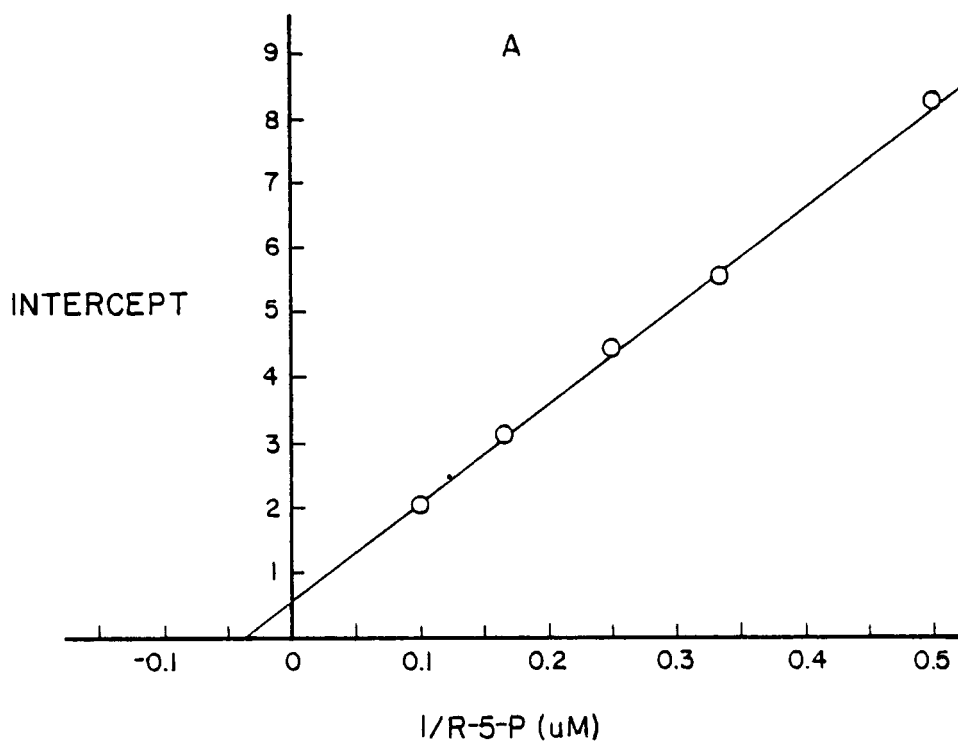


Figure 11.

Figure 12. Secondary Plots of Figure 10, Part B Results (KC62).

The intercept plot (Part A) is the $1/V$ intercept values from Figure 10, Part B versus $1/R-5-P$ concentration. The slope plot (Part B) is the slope values from Figure 10, Part B versus $1/R-5-P$ concentration.

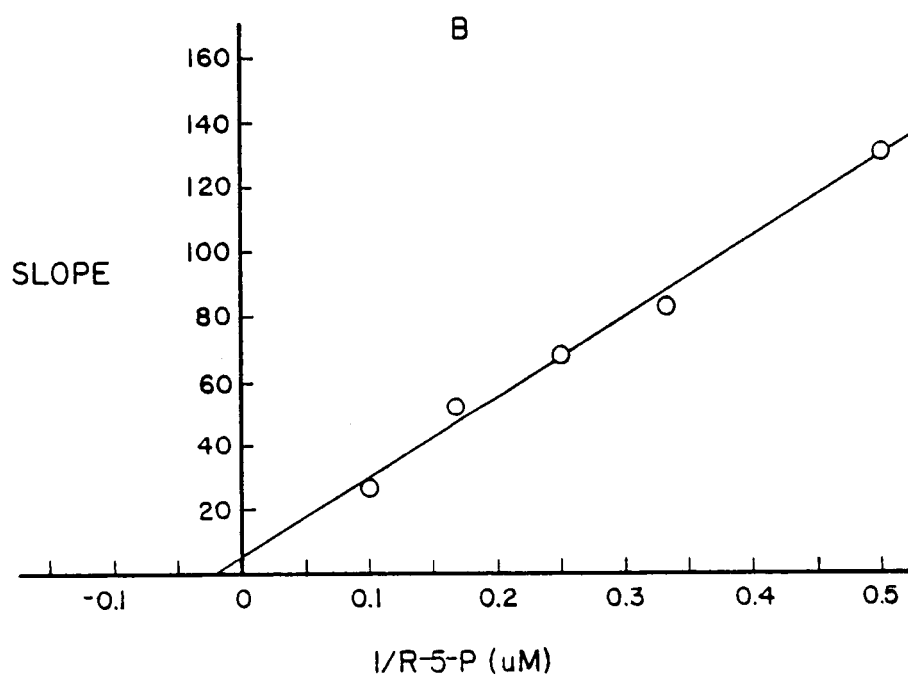
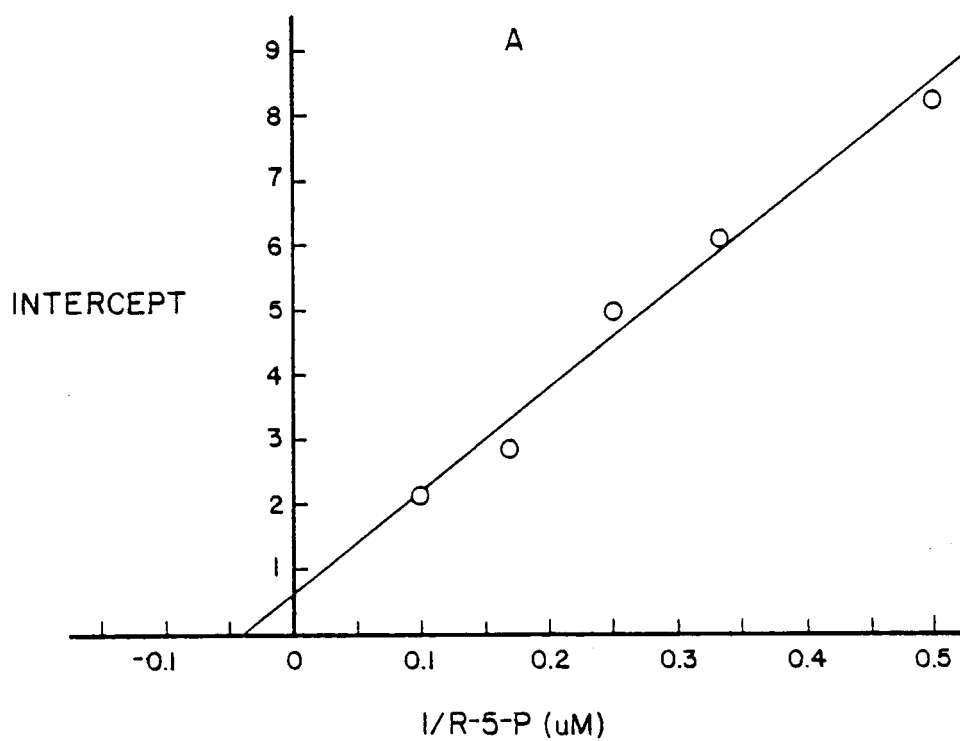


Figure 12.

TABLE IV.
APPARENT KINETIC PARAMETERS FOR PURIFIED PRPP-SYNTHETASES

Trial	V-79A		KC62	
	K_m ATP ^a	K_m R-5-pa	K_m ATP ^a	K_m R-5-pa
I	9.74	22.68	10.82	25.62
II	13.02	26.93	7.63	23.81
Average ^b	11.38 ± 1.64	24.80 ± 2.12	9.23 ± 1.60	24.71 ± 0.91

a K_m has the units μ moles/liter.

b Average represents the mean $\pm$ 1 S.D.

Discussion

Double reciprocal plots of the initial velocity data for both the V-79A and KC62 enzyme are intersecting and suggest a sequential reaction mechanism for both enzymes. Table IV results suggest that no significant differences exist in the K_m values of the PRPP-synthetases of V-79A and KC62. This was an expected finding since V-79A and KC62 K_m values determined with 110,000 x G supernatant preparations were found to be very similar. The K_m values determined for purified PRPP-synthetases shown in Table IV are similar to those determined for PRPP-synthetases in 110,000 x G supernatants shown in Table I. This suggests that the catalytic activities of the enzymes are not adversely affected by the purification procedure.

Several investigators have done similar initial velocity studies with purified PRPP-synthetases from human erythrocytes and Salmonella typhimurium. Fox and Kelly (35), and Becker et al. (20) have examined purified PRPP-synthetases from human erythrocytes. Results from initial velocity and inhibitor studies with a 400 fold purified PRPP-synthetase lead Fox and Kelly to conclude that the PRPP-synthetase reaction mechanism was an Ordered Bi Bi mechanism in which R-5-P binds first and PRPP is released last. K_m values for ATP and R-5-P were 14 μM and 33 μM , respectively. Becker, using a 4,500 fold purified erythrocyte enzyme determined the K_m values for ATP and R-5-P to be 30 μM and 410 μM , respectively. Becker's K_m value for R-5-P is much larger than that reported by Fox and Kelly. Becker attributed this difference to using a higher and wider range of variable substrate concentrations than Fox and Kelly.

Switzer (18,53) has examined in detail the kinetic properties of a 450 fold purified PRPP-synthetase from Salmonella typhimurium. Switzer proposed that this PRPP-synthetase had an Ordered Bi Bi mechanism in which ATP binds first and PRPP is released last. K_m values for ATP and R-5-P were determined to be 46 μM and 160 μM , respectively. Roth et al. (34) have determined the K_m values of a 1,000 fold purified rat liver PRPP-synthetase. In these studies, however, the PRPP-synthetase reaction was made pseudo-first order. The K_m values determined for ATP and R-5-P were 220 μM and 290 μM , respectively.

D. Inhibitor Studies

Materials and Methods

The procedure by which nucleotide inhibitor studies of purified V-79A and KC62 PRPP-synthetase were performed was identical to that outlined in Chapter III, Initial Velocity Studies, Materials and Methods, except that nucleotide inhibitors were included in the assay mixture. Three different nucleotide inhibitors (AMP, GMP, and XMP) were tested individually each at three different concentrations. Approximately 0.5 μg of protein having a specific activity of at least 1,650 units/mg (400 fold purification) was used for each determination. All assays were 6 minutes in duration. The resulting data was analyzed using Dixon plots or Cornish-Bowden plots and the apparent K_i values were determined graphically. The apparent K_i value was determined by the point of intersection on the horizontal axis of a line drawn through the intersection of the family of resulting lines and perpendicular to the horizontal axis.

Results

Saturation of the V-79A or KC62 enzyme by either ATP or R-5-P in the presence of three different concentrations of either AMP, XMP or GMP has been examined. Typical results from some of these inhibitor studies are shown in Figures 13 through 20. Figures 13, 15, 17 and 19 are for the V-79A enzyme and Figures 14, 16, 18 and 20 are for the KC62 enzyme. Part A of each figure is a double reciprocal plot of the data while Part B is a secondary plot of the same data. For any given inhibitor and variable substrate, the resulting type of inhibition pattern was the same for the V-79A and KC62 enzyme. In all but one case the double reciprocal plots of the data resulted in a family of lines intersecting to the left of the vertical axis, suggesting noncompetitive inhibition. In these cases, the apparent K_i values were determined graphically from the Dixon plots. The single exception was the saturation of both the V-79A and KC62 enzyme by R-5-P in the presence of GMP. Figure 19 for the V-79A enzyme and Figure 20 for the KC62 enzyme show that under these conditions the double reciprocal plots of the data resulted in a family of parallel lines, suggesting uncompetitive inhibition. K_i values in these cases were determined graphically from Cornish-Bowden plots.

A summary of the apparent K_i values determined in these studies is shown in Table V. In some instances two different apparent K_i values are given for a particular inhibitor-variable substrate combination. These K_i values resulted from two different determinations using different purified preparations of the enzyme. For all three inhibitors tested, the apparent K_i values of the KC62 enzyme were less than those for the V-79A enzyme. V-79A's apparent K_i values are 3.8 fold and 5.4 fold greater for AMP and XMP, respectively, than those of the KC62 enzyme when ATP was the variable substrate.

Figure 13. AMP Inhibition of V-79A PRPP-Synthetase Activity at Variable ATP Concentration.

Part A is a double reciprocal plot of initial velocity at variable ATP concentrations from 5 to 20 μM in the presence of 500 μM R-5-P and 0 ($\circ$), 25 (Δ), 50 ($\square$), and 100 μM (∇) AMP. Velocity is in nanomoles/min. Part B is a Dixon Plot ($1/V$ versus AMP concentration) of Part A data.

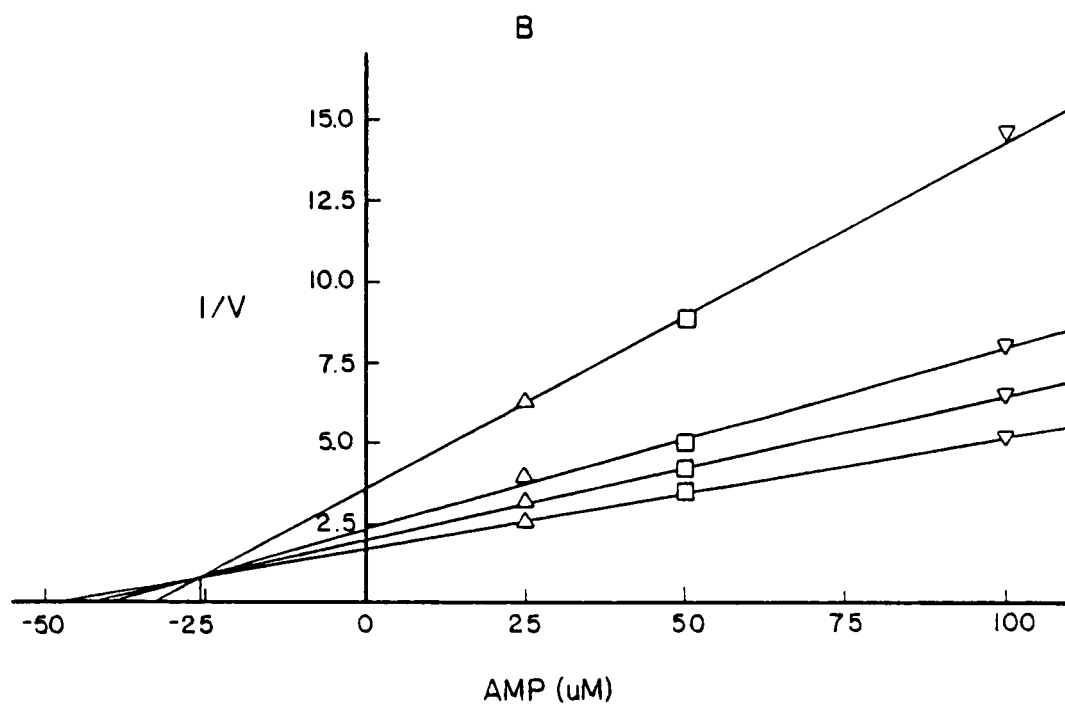
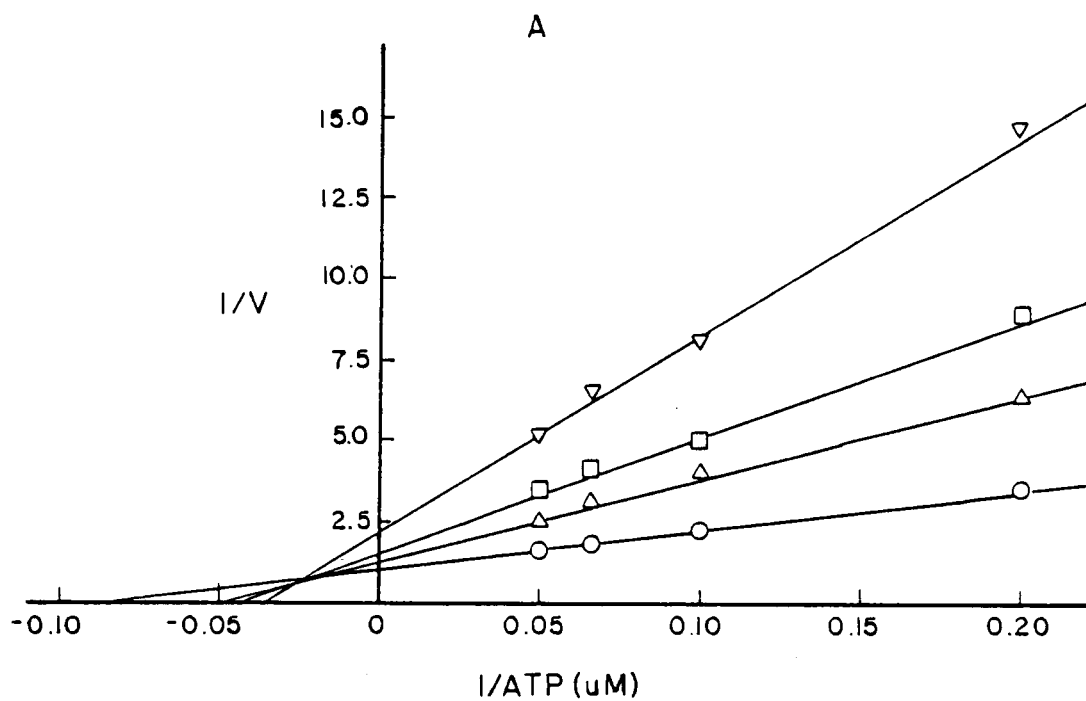


Figure 13.

Figure 14. AMP Inhibition of KC62 PRPP-Synthetase Activity at Variable ATP Concentration.

Part A is a double reciprocal plot of initial velocity at variable ATP concentrations from 5 to 20 μM in the presence of 500 μM R-5-P and 0 (O), 25 (Δ), 50 ($\square$), and 100 μM (∇) AMP. Velocity is in nanomoles/min. Part B is a Dixon Plot ($1/V$ versus AMP concentration) of Part A data.

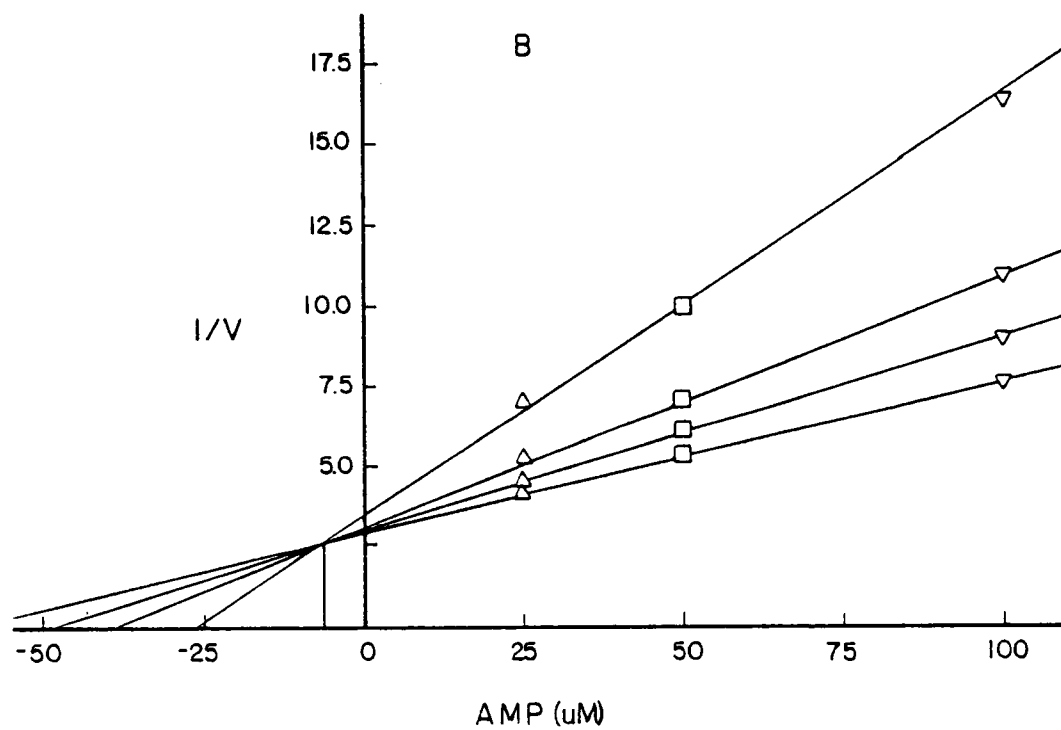
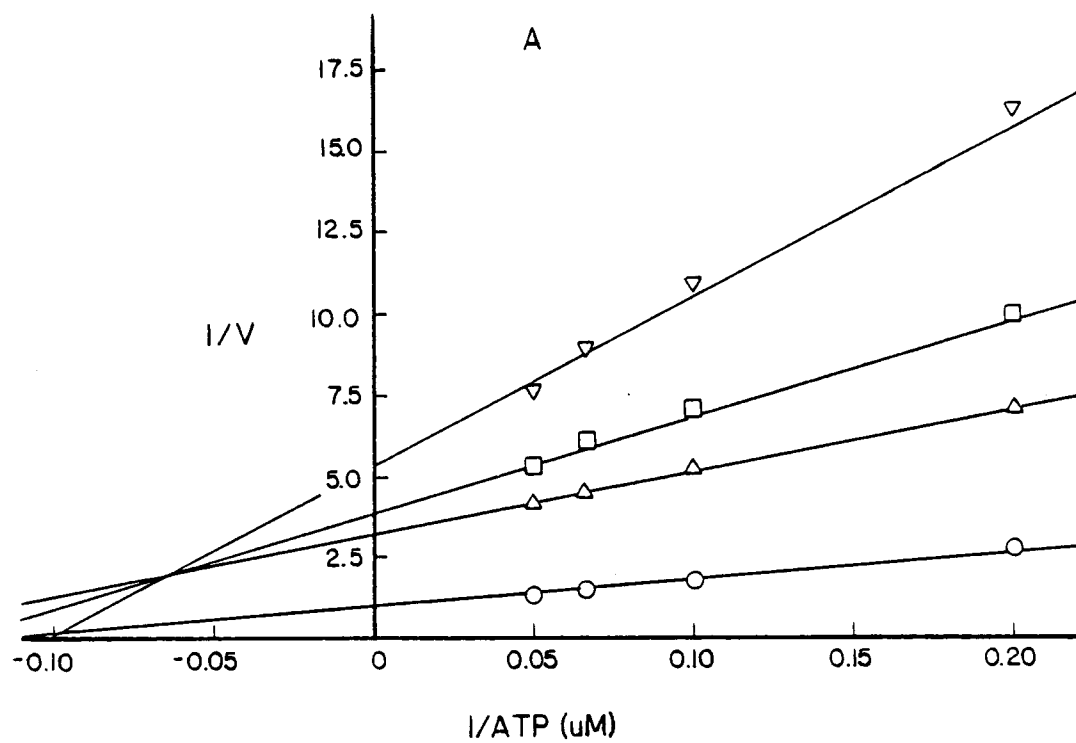


Figure 14.

Figure 15. XMP Inhibition of V-79A PRPP-Synthetase Activity at Variable ATP Concentration.

Part A is a double reciprocal plot of initial velocity at variable ATP concentrations from 5 to 20 μM in the presence of 500 μM R-5-P and 0 (O), 50 (Δ), 100 ($\square$), and 250 μM (∇) XMP. Velocity is in nanomoles/min. Part B is a Dixon Plot ($1/V$ versus XMP concentration) of Part A data.

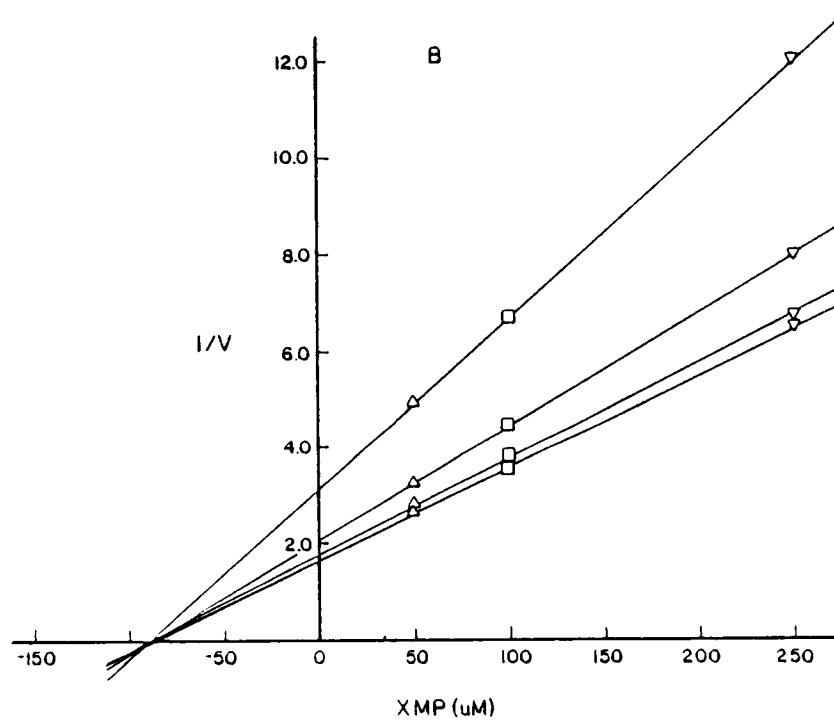
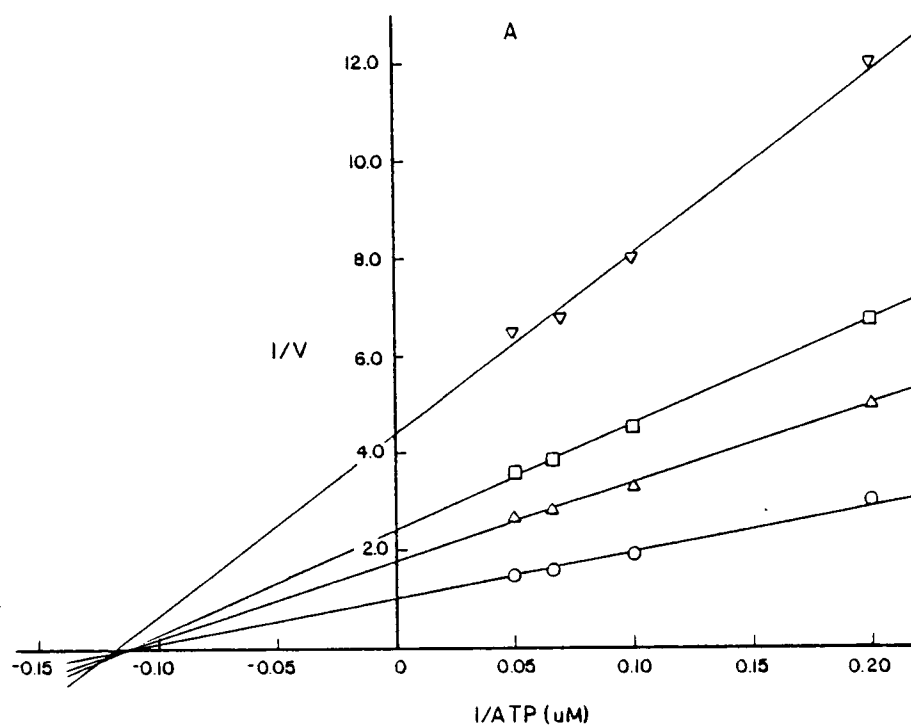


Figure 15.

Figure 16. XMP Inhibition of KC62 PRPP-Synthetase Activity at Variable ATP Concentration.

Part A is a double reciprocal plot of initial velocity at variable ATP concentrations from 5 to 20 μM in the presence of 500 μM R-5-P and 0 (O), 50 (Δ), 100 ($\square$), and 250 μM (∇) XMP. Velocity is in nanomoles/min. Part B is a Dixon Plot ($1/V$ versus XMP concentration) of Part A data.

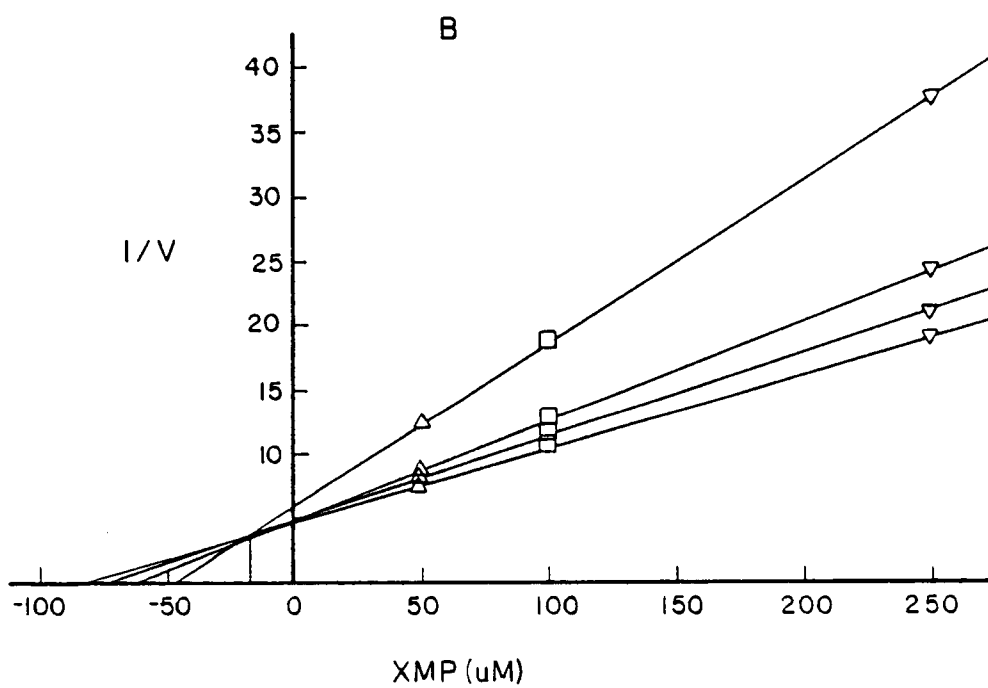
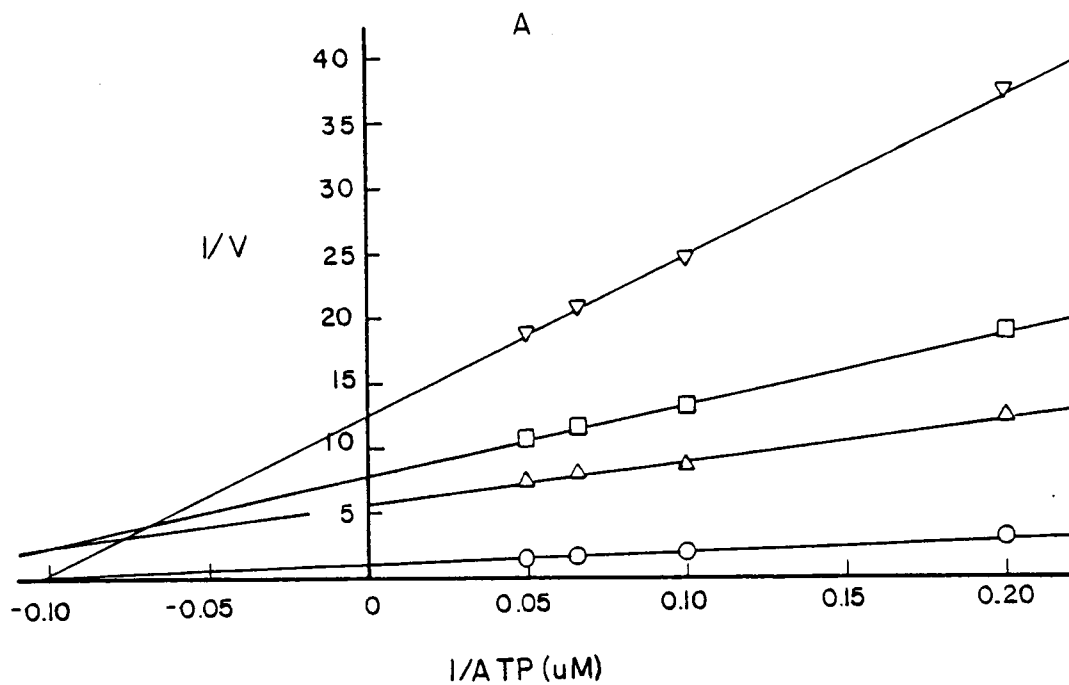


Figure 16.

Figure 17. XMP Inhibition of V-79A PRPP-Synthetase Activity at Variable R-5-P Concentration.

Part A is a double reciprocal plot of initial velocity at variable R-5-P concentrations from 10 to 40 μM in the presence of 500 μM ATP and 0 (O), 50 (Δ), 100 ($\square$), and 250 μM (∇) XMP. Velocity is in nanomoles/min. Part B is a Dixon Plot ($1/V$ versus XMP concentration) of Part A data.

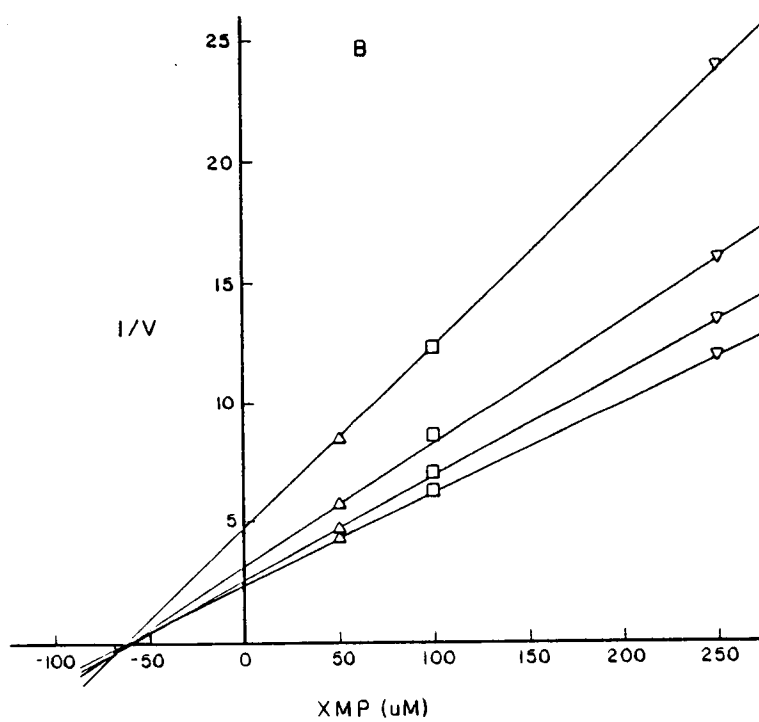
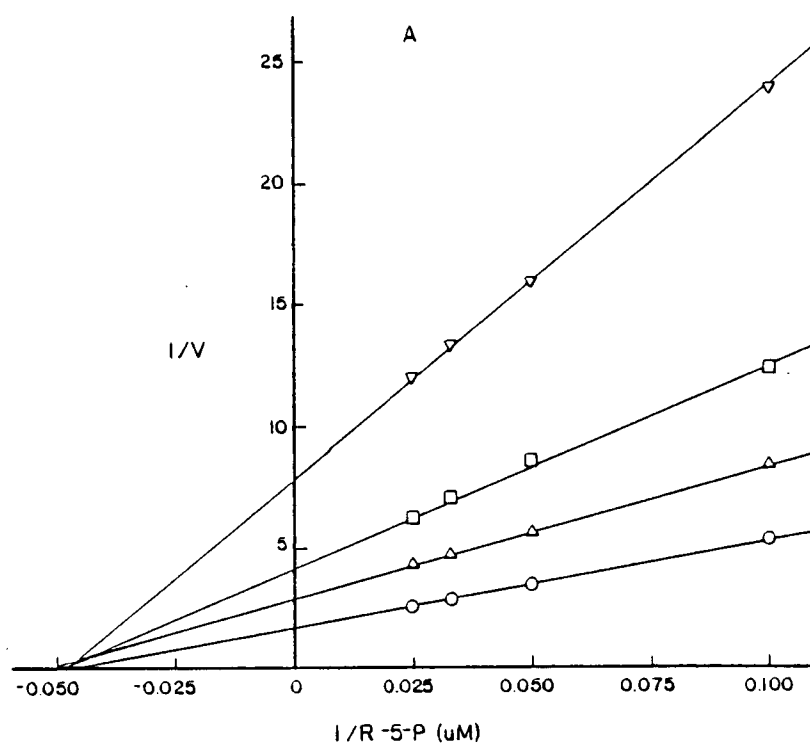


Figure 17.

Figure 18. XMP Inhibition of KC62 PRPP-Synthetase Activity at Variable R-5-P Concentration.

Part A is a double reciprocal plot of initial velocity at variable R-5-P concentrations from 10 to 40 μM in the presence of 500 μM ATP and 0 ($\circ$), 50 (Δ), 100 ($\square$), and 250 μM (∇) XMP. Velocity is in nanomoles/min. Part B is a Dixon Plot ($1/V$ versus XMP concentration) of Part A Data.

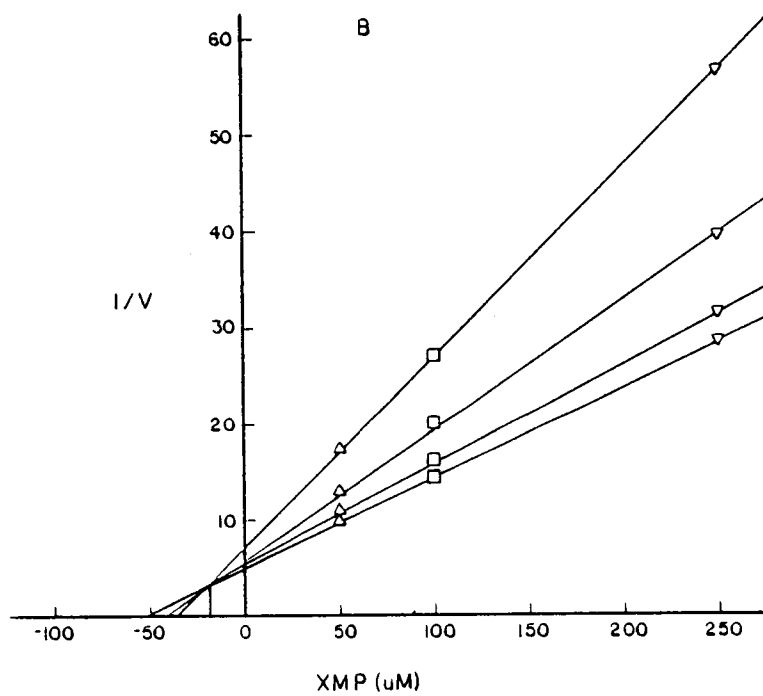
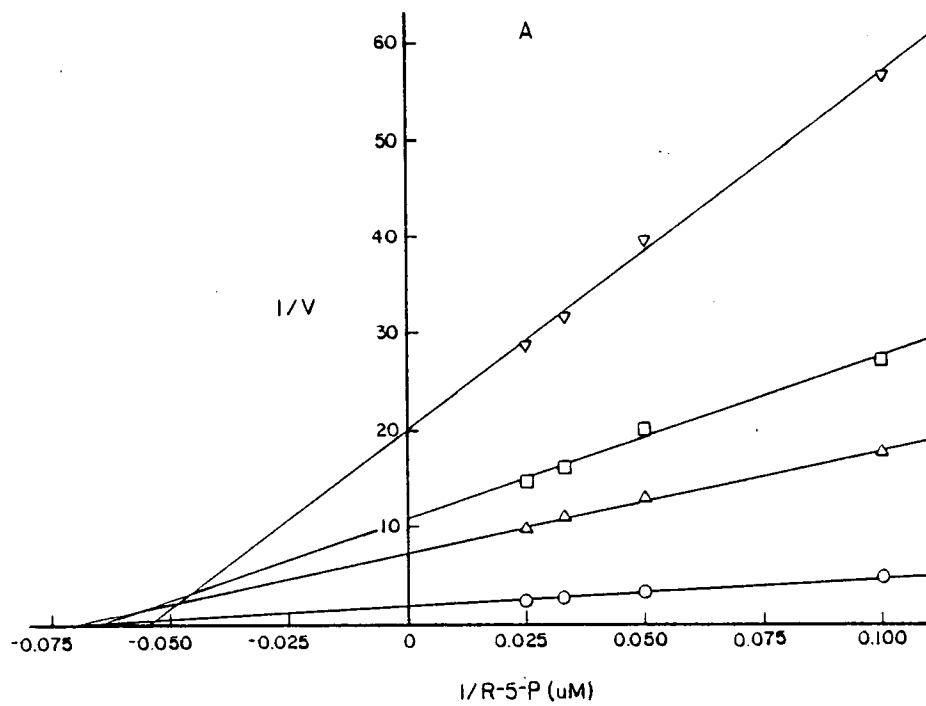


Figure 18.

Figure 19. GMP Inhibition of V-79A PRPP-Synthetase Activity at Variable R-5-P Concentration.

Part A is a double reciprocal plot of initial velocity at variable R-5-P concentrations from 10 to 40 μM in the presence of 500 μM ATP and 0 ($\circ$), 250 (Δ), 500 ($\square$), and 750 μM (∇) GMP. Velocity is in nanomoles/min. Part B is a Cornish-Bowden Plot (S/V versus GMP concentration) of Part A data.

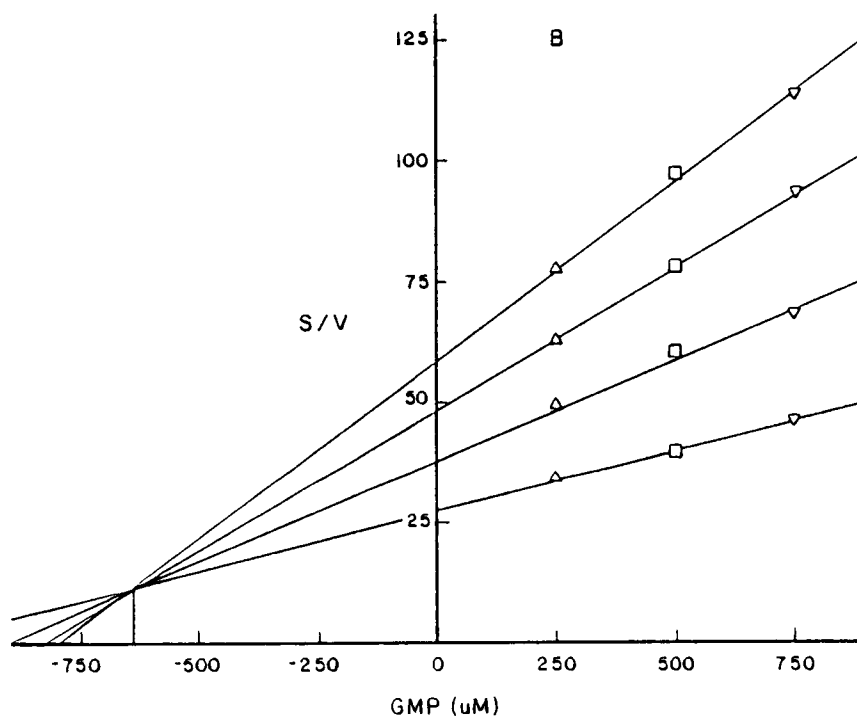
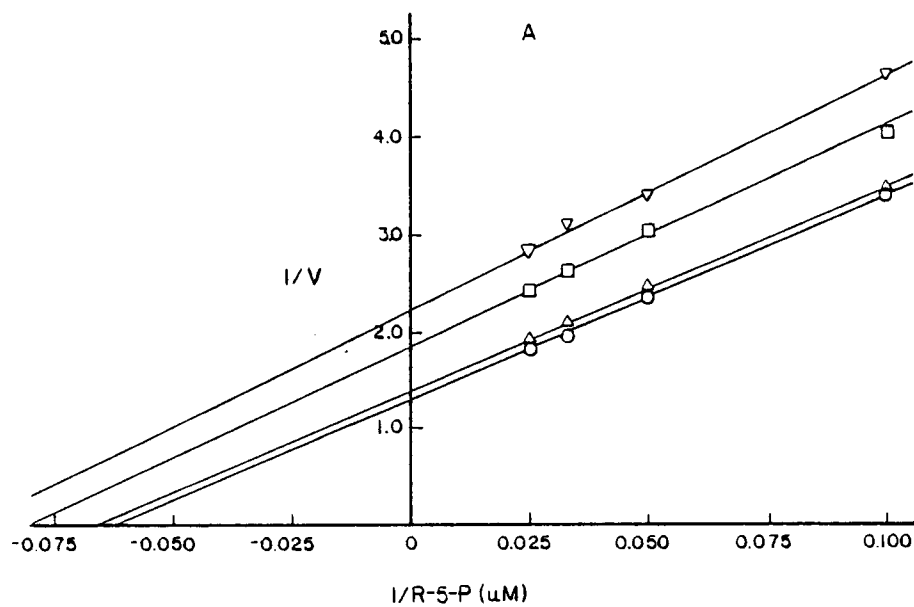


Figure 19.

Figure 20. GMP Inhibition of KC62 PRPP-Synthetase Activity at Variable R-5-P Concentration.

Part A is a double reciprocal plot of initial velocity at variable R-5-P concentrations from 10 to 40 μM in the presence of 500 μM ATP and 0 (O), 250 (Δ), 500 ($\square$), and 750 μM (∇) GMP. Velocity is in nanomoles/min. Part B is a Cornish-Bowden Plot (S/V versus GMP concentration) of Part A data.

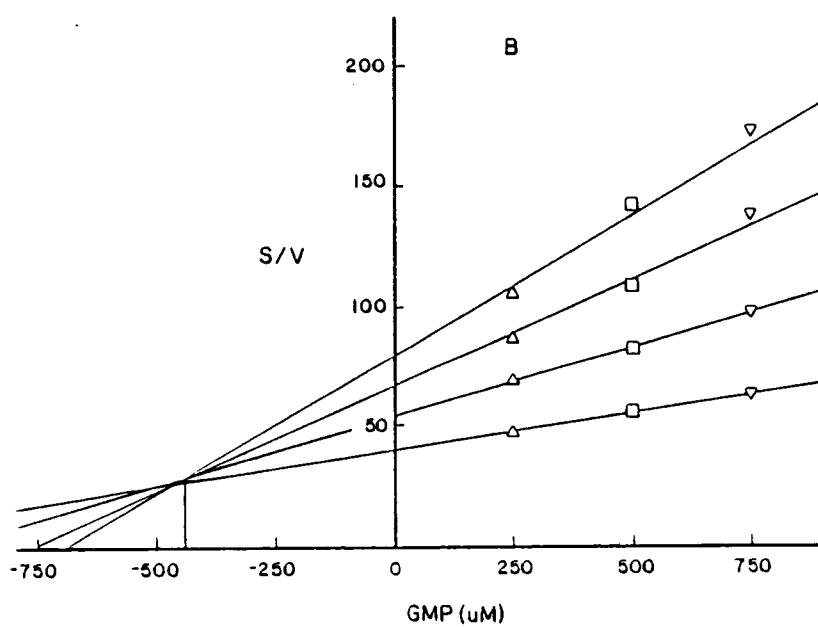
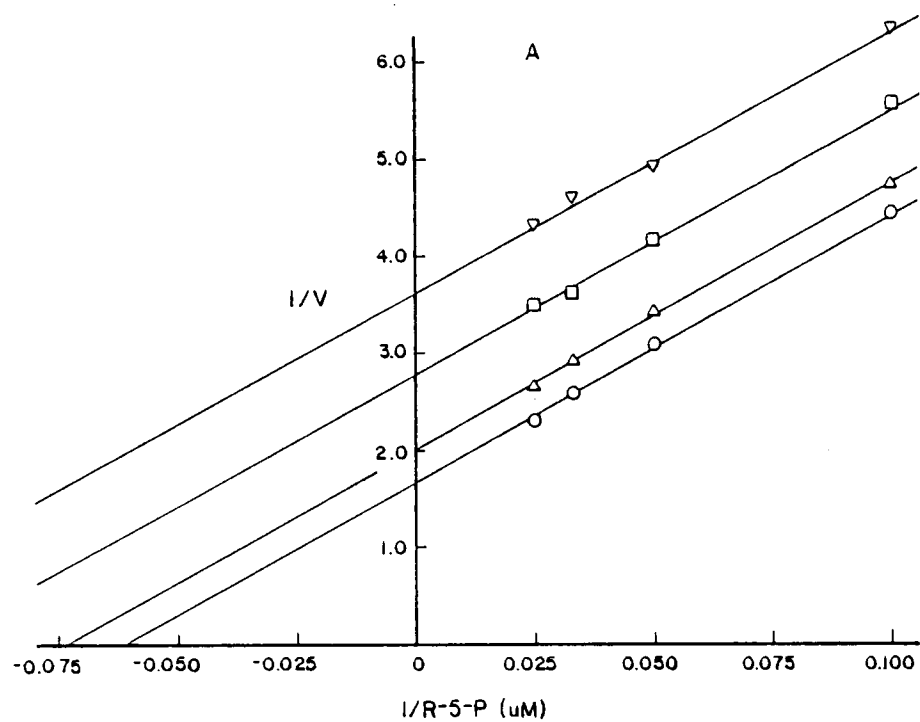


Figure 20.

TABLE V.
APPARENT INHIBITOR CONSTANTS FOR PURIFIED PRPP-SYNTHETASES

Inhibitor	Variable Substrate	V79A K _i ^a	KC62 K _i ^a	Type of Inhibition
AMP	R-5-P ATP	620.0 22.0, 26.0	415.0 6.5, 6.0	Noncompetitive Noncompetitive
GMP	R-5-P ATP	640.0 54.0, 54.6	440.0 33.0, 30.5	Uncompetitive Noncompetitive
XMP	R-5-P ATP	68.8 93.8	18.8 17.5	Noncompetitive Noncompetitive

^aK_i has the units μ moles/liter.

V-79A's apparent K_i value is 3.7 fold greater for XMP than that of KC62's when R-5-P was the variable substrate. Clearly, in these cases KC62's apparent K_i values are significantly less than those of V-79A's.

Discussion

The inhibitor studies with the 110,000 x G supernatant preparations provided preliminary evidence that the KC62 PRPP-synthetase was more sensitive to nucleotide inhibition than its V-79A counterpart. The K_i values determined for the purified enzymes from V-79A and KC62 verified those preliminary findings. While decreases in KC62's PRPP-synthetase K_i values are slight in some cases, in others a large and significant decrease is apparent. The results of the K_i value determinations support the idea that KC62's PRPP-synthetase is a mutant enzyme form which possesses altered regulatory properties with respect to nucleotide inhibitors.

Mutant forms of human erythrocyte and fibroblast PRPP-synthetase reported by other investigators have previously been discussed (see Chapter III, Inhibitor Studies, Discussion). Additionally, Green and Martin (26) have reported a mutant form of PRPP-synthetase from a mutagenized, cultured rat hepatoma cell line that overproduces purines. The mutant enzyme had normal catalytic properties, but a decreased sensitivity to inhibition by purine and pyrimidine nucleotides. The types of inhibition exerted by AMP, ADP and TDP on the wild type enzyme had been maintained, but values for K_i had been increased by factors of 10, 17.5 and 5, respectively. To date, there have been no reports of mutant PRPP-synthetase with increased sensitivity to nucleotide inhibitors. However, in lower organisms mutant enzyme forms with increased sensitivity to feedback inhibitors have been reported. Springer et al. (54) have isolated several mutant strains of Salmonella typhimurium whose

threonine deaminase had normal catalytic properties but had increased sensitivity to the feedback inhibitor isoleucine. Pabst et al. (55) have isolated several mutant strains of Escherichia coli whose "anthranilate aggregate" (anthranilate synthetase-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase enzyme aggregate) had normal catalytic properties, but had an increased sensitivity to the feedback inhibitor tryptophan. These reports suggest that mutant enzyme forms with normal catalytic properties and increased sensitivity to feedback inhibitors are possible.

Switzer (18,53), using initial velocity, inhibitor, and isotope exchange studies, has extensively examined the kinetic properties of a purified PRPP-synthetase from Salmonella typhimurium. Fox and Kelly (35), and Becker et al. (20) have examined the kinetic properties of purified PRPP-synthetase preparations from human erythrocytes. The results of their studies led both Switzer, and Fox and Kelly to propose sequential Ordered Bi Bi reaction mechanisms for the respective PRPP-synthetases examined. A major difference in the proposed reaction mechanisms was the order of addition of the substrates. Switzer proposed that the leading substrate for the Salmonella typhimurium enzyme was ATP, while Fox and Kelly proposed that the leading substrate for the erythrocyte enzyme was R-5-P. In the studies of both Switzer, and Fox and Kelly assignment of the order of addition of the substrates and order of release of products was made, in part, on the basis of adherence to specific predicted inhibition patterns proposed by Cleland (56,57,58). In the case of Switzer's studies, further support for the proposed order of the mechanism was provided by isotope exchange studies.

The results of the initial velocity studies in which nucleotides were absent suggested a sequential mechanism for the PRPP-synthetases from V-79A and

KC62. The results of the K_i determinations furnish some additional information concerning the reaction mechanism of the PRPP-synthetases from V-79A and KC62. Conclusions that can be drawn from these studies are limited, in part, since the coupled assay system employed did not allow assessment of product inhibition by PRPP.

In the K_i determinations with AMP as the inhibitor, the concentration of either substrate was varied while the cosubstrate was maintained at a saturating concentration. If one assumes that AMP acts as a product inhibitor (as have Fox and Kelly, and Switzer) some conclusions can be made. If AMP is the penultimate or last product released from the enzyme, AMP inhibition should give linear double reciprocal plots of a noncompetitive pattern if the enzyme proceeds by a strict Random Bi Bi mechanism regardless of which substrate was varied (with the cosubstrate saturating) (59). In the inhibitor studies AMP did result in noncompetitive inhibition patterns in all cases. However, replots of $1/V$ intercept or slope versus inhibitor concentration would be hyperbolic if the mechanism was a strict Random Bi Bi mechanism (59). Plots of $1/V$ intercept versus inhibitor concentration and slope versus inhibitor concentration were found to be linear in all cases for the V-79A and KC62 enzyme. Further, if the enzyme proceeded by such a mechanism the double reciprocal plots of the initial velocity with both substrates at subsaturating concentrations would be nonlinear (59). Both substrates were at subsaturating concentrations in the initial velocity studies done to determine K_m values and the double reciprocal plots were linear. If the mechanism was a Rapid Equilibrium Bi Bi mechanism or some derivative of a Rapid Equilibrium Bi Bi mechanism, AMP would produce no inhibition or competitive inhibition with respect to at least one substrate (59). This was not found to be the case.

The AMP inhibition patterns (all noncompetitive) for the V-79A and KC62 PRPP-synthetases suggest that the enzyme does not proceed by any type of random mechanism.

Still assuming AMP to be a product inhibitor and keeping in mind that in all cases the cosubstrate was saturating, one can analyze whether the AMP inhibition patterns are those predicted for any possible Steady State Ordered Bi Bi mechanism. If one assumes that ATP is the leading substrate and that AMP is the final product released from the enzyme, then AMP inhibition should be competitive when ATP is the variable substrate and no inhibition should occur when R-5-P is the variable substrate (59). If one assumes that R-5-P is the leading substrate and that AMP is the final product released from the enzyme, then AMP inhibition should be competitive when R-5-P is the variable substrate and no inhibition should occur when ATP is the variable substrate (59). Neither of these schemes results in inhibition patterns consistent with the actual results which showed AMP to be noncompetitive with respect to both substrates when the cosubstrate was saturating. If one assumes that ATP is the leading substrate and that AMP is the penultimate product released from the enzyme, then AMP inhibition should be uncompetitive when ATP is the variable substrate and noncompetitive when R-5-P is the variable substrate (59). If one assumes that R-5-P is the leading substrate and that AMP is the penultimate product released from the enzyme, then AMP inhibition should be uncompetitive when R-5-P is the variable substrate and noncompetitive when ATP is the variable substrate (59). Neither of these two schemes is completely consistent with the actual AMP inhibition patterns since in no case did AMP result in uncompetitive inhibition patterns. Therefore, it is not possible to state unequivocally that the V-79A and KC62 enzyme proceed by an Ordered

Bi Bi mechanism. Obviously, it is not possible to assign the order of addition of substrates or order of release of products for these PRPP-synthetases.

Nonadherence to predicted inhibition patterns is not unique to the PRPP-synthetases from V-79A and KC62 cells. Roth et al. (34), using a highly purified PRPP-synthetase from rat liver have observed results similar to those found for the PRPP-synthetases from V-79A and KC62. AMP inhibition was noncompetitive with respect to both substrates when cosubstrate was saturating. Under similar assay conditions, Switzer (25) found this to be the case for the Salmonella typhimurium enzyme as well. Green and Martin (26), using a crude preparation from a rat hepatoma cell line have also examined AMP inhibition. AMP inhibition appeared to be competitive with respect to ATP and uncompetitive with respect to R-5-P when respective cosubstrate concentrations were saturating. Thus in the studies by Roth, Green and Martin, and Switzer, AMP inhibition patterns are not fully consistent with those predicted for any Ordered Bi Bi mechanism.

Both Fox and Kelly (35), and Becker et al. (20), using purified human erythrocyte PRPP-synthetases, have found AMP to be a noncompetitive inhibitor with respect to both substrates when subsaturating concentrations of cosubstrate were present. These inhibition patterns one would predict if AMP acts as a product inhibitor and if the order of addition of the substrates and order of release of products is that proposed by Fox and Kelly. However, the fact that AMP inhibition was found to be noncompetitive with respect to both substrates in the studies of Roth et al. (34), Switzer (25) and those of the V-79A and KC62 enzymes under assay conditions where these inhibition patterns would not be predicted, strongly suggests that AMP's primary inhibitory mode is not that of a product inhibitor, but as a feedback inhibitor which binds to

an allosteric site. Green and Martin have made a similar suggestion (26). AMP is not only an immediate product of the PRPP-synthetase reaction, but an end product of the pathways responsible for purine nucleotide production. The PRPP-synthetase reaction is known to play a critical regulatory role in this biosynthetic sequence and evidence is abundant that this reaction is subject to feedback regulation by a variety of nucleotide end products. E. R. Stadtman states in reference to feedback regulation (60):

When apparently noncompetitive kinetics are observed it seems that binding of the end product to an inhibitor site (not the catalytic site) on the enzyme results in the induction or stabilization of a conformational state that has a lower catalytic potential, ...

Studies by Green and Martin (26), and Sperling et al. (45,46) of mutant forms of PRPP-synthetase support the idea that AMP inhibits by binding to an allosteric site. In both studies the mutant PRPP-synthetases had normal catalytic activity but substantially decreased sensitivity to inhibition by AMP. The studies of the V-79A and KC62 enzymes further substantiate this idea. The mutant enzyme has normal catalytic activity, but increased sensitivity to inhibition by AMP.

It is reasonable to believe that GMP and XMP also bind to allosteric sites and act as feedback inhibitors of the PRPP-synthetases of V-79A and KC62. It has been suggested by Fox and Kelly (35) that in the case of human erythrocyte PRPP-synthetase, that all feedback inhibitors bind to a single type of allosteric site. In determination of K_i values for the V-79A and KC62 enzymes only one inhibitor at a time was tested, so it is not possible to address the question of whether a single type of allosteric site exists in these PRPP-synthetases. Only one investigation has reported a K_i value for GMP. Fox and Kelly (35), using purified PRPP-synthetase from human erythrocytes, found GMP to be noncompetitive with respect to ATP (at subsaturating

cosubstrate concentrations) with a K_i value of 2.0 mM. To date, there have been no published K_i values or reports of the type of inhibition exerted by XMP on PRPP-synthetase. All three nucleotide inhibitors tested on V-79A and KC62 enzyme resulted in noncompetitive inhibition, except the uncompetitive inhibition of GMP when R-5-P was the variable substrate. The reason for GMP's uncompetitive inhibition under these conditions is unknown.

E. Phosphate Activation

Materials and Methods

PRPP-synthetase having a specific activity of at least 1,650 units/mg (400 fold purification) was placed in a Spectrapor 2 membrane and dialyzed for 24 hours against 4,000 to 5,000 volumes of 4°C HEPES buffer (pH 7.5) containing 6 mM $MgCl_2$, 60 μ M ATP and 0.1 mM DTT. PRPP-synthetase activity at various Na_3PO_4 concentrations was determined by the procedure outlined in Chapter III, Initial Velocity Studies, Materials and Methods. All assays were 6 minutes in duration and 0.3 μ g of protein was used for each determination. The assay mixture had a final volume of 500 μ l and contained the following:

1. 50 μ l of 5.0 mM ATP in H_2O
2. 50 μ l of 5.0 mM R-5-P in H_2O
3. 20 μ l of 0.41 mM [Carboxy- ^{14}C] orotic acid (0.01 μ Ci/ μ l) in H_2O
4. 80 μ l of 0.5 mg/80 μ l OPRT-ODC in 50 mM HEPES buffer (pH 7.5) containing 6 mM $MgCl_2$
5. 50 μ l of 0.320 M HEPES buffer (pH 7.5) containing 38.4 mM $MgCl_2$
6. 150 μ l of various concentrations of Na_3PO_4 (pH 7.4) buffer
7. 100 μ l diluted, dialyzed enzyme in 50 mM HEPES buffer (pH 7.5) containing 6 mM $MgCl_2$, 60 μ M ATP, and 0.1 mM DTT.

The final concentrations in the assay mixture were 50 mM HEPES, 500 μ M ATP, 500 μ M R-5-P, 16.4 μ M orotic acid, 6 mM MgCl_2 and Na_3PO_4 concentrations of 0.5 to 50 mM.

Results

Two separate determinations of phosphate activation for the V-79A and KC62 PRPP-synthetases were done. Both the V-79A and the KC62 enzyme reached maximal velocity at approximately 30 mM phosphate. The phosphate activation values for the two separate trials for each enzyme type were expressed as a percentage of the maximal velocities achieved and averaged. Figure 21 shows the resulting phosphate activation curves for both the V-79A and KC62 enzymes. Figure 21 shows that there is no difference in either the pattern or degree of phosphate activation between the two enzymes. Half maximal velocity for both enzymes was achieved at approximately 1.25 mM phosphate. Preliminary studies indicated that at phosphate concentrations above 50 mM, both the V-79A and KC62 enzymes showed a similar, steady decrease in activity with increasing phosphate concentrations up to 150 mM.

Discussion

To date, PRPP-synthetases from all sources examined have been shown to have an absolute requirement for inorganic phosphate which acts as an allosteric activator. In those studies in which the pattern of phosphate activation was determined, enzyme activity responded hyperbolically to increasing phosphate concentrations. This general pattern of phosphate activation was also observed for PRPP-synthetase from V-79A and KC62 as shown in Figure 21. Studies by other investigators with PRPP-synthetases from human erythrocytes (17), Ehrlich ascites tumor cells (24) and rat liver

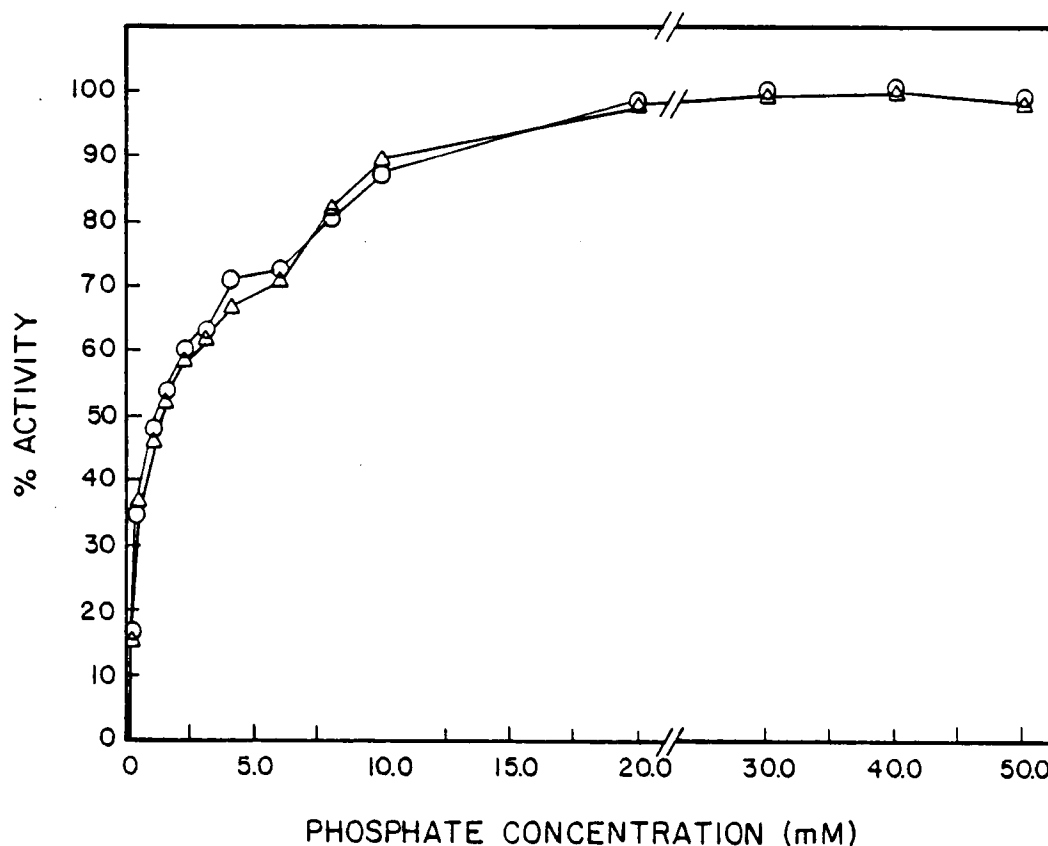


Figure 21. Phosphate Activation of PRPP-Synthetase.

PRPP-synthetase having a specific activity of at least 1,650 units/mg was dialyzed for 24 hours against 4,000 to 5,000 volumes of 4°C HEPES buffer (pH 7.5) containing 6 mM MgCl_2 , 60 μM ATP and 0.1 mM DTT. Phosphate was added back at the concentrations shown and the enzyme was assayed at final ATP and R-5-P concentrations of 500 μM each. V-79A (O), KC62 (Δ).

(19) have demonstrated that loss of activity as a result of phosphate removal is reversible by readdition of phosphate. In the case of PRPP-synthetase from Salmonella typhimurium, however, phosphate removal leads to an irreversible loss of activity (18). The phosphate activation studies with V-79A and KC62 PRPP-synthetases demonstrate that both enzymes have an absolute requirement for phosphate for activity, and that loss of activity resulting from phosphate removal was reversible by readdition of phosphate.

The results of the phosphate activation studies of the V-79A and KC62 PRPP-synthetases indicated that there was no difference in either the pattern or degree of phosphate activation for the V-79A and KC62 enzymes. Phosphate activation studies of Km, Vmax and Ki mutants of human erythrocyte PRPP-synthetase have been done by Becker et al. (44), Becker et al. (20,41,42) and Sperling et al. (45), respectively. In each of these studies the pattern and degree of phosphate activation of the mutant enzyme was similar to that of the wild type enzyme. The results of these studies with mutant forms of erythrocyte PRPP-synthetase as well as those with the mutant PRPP-synthetase from KC62, suggest that structural alterations affecting the catalytic and regulatory properties are possible without affecting the mechanism by which phosphate activates PRPP-synthetase.

The phosphate concentrations required for half maximal and maximal activity for a variety of eukaryotic PRPP-synthetases have been reported. For various preparations of human erythrocyte PRPP-synthetase, phosphate concentrations of 0.52 mM to 4.5 mM have been reported for half maximal activity, and 32 mM to 50 mM for maximal activity (20,41,42,44). A 1,000 fold purified rat liver PRPP-synthetase had values of 4.3 mM and 100 mM phosphate for half maximal and maximal activity, respectively (34). Ehrlich

ascites tumor cell PRPP-synthetase had values of 3.3 mM and 50 mM phosphate for half maximal and maximal activity, respectively (24). The phosphate concentrations required for half maximal and maximal activity for the PRPP-synthetases for V-79A and KC62 fall within the ranges for PRPP-synthetases from other sources.

CHAPTER V

NUCLEOTIDE POOLS OF V-79A AND KC62 CELLS

A. High Pressure Liquid Chromatography

Materials and Methods

The cell extracts on which HPLC (high pressure liquid chromatography) was performed were prepared from the cells contained on five 100 mm culture plates each of identical cell number. After removal of LAC medium, each of the five culture plates of cells was rapidly, twice washed with 2 ml of 4°C Hanks' balanced salt solution. To each plate was added 1 ml of 4°C 0.4 M PCA (perchloric acid) containing either 0.23 μ M [^{3} H-(G)] hypoxanthine (2.9 Ci/mmol) (New England Nuclear) or 4.0 μ M [^{8} - ^{14} C]ATP (50.1 mCi/mmol) (New England Nuclear). A rubber policeman was used to scrape the cells loose from the plates and a known volume of the PCA mixture pooled from the five plates was centrifuged at 120 x G for 5 minutes at 4°C. A known volume of the PCA supernatant was transferred to another test tube and neutralized to pH 7.0 with 4°C 10 N KOH. The neutralized PCA supernatant was centrifuged at 120 x G for 5 minutes at 4°C to pellet the KClO₄. A known volume of the neutralized supernatant was transferred to another test tube and its volume decreased by 3 to 4 fold by evaporation with a 4°C stream of N₂. A 100 to 150 μ l sample of this concentrated material was subjected to HPLC to determine nucleotide concentrations.

A sixth 100 mm culture plate identical in cell number to those used for preparation of cell extract for HPLC was used to determine the protein content per plate. After removal of LAC medium, the cells were rapidly, twice washed with 2 ml of Hanks' balanced salt solution. The cellular material was removed

from the culture plate with four 1 ml portions of 0.2 N NaOH and one 1 ml portion of H₂O. The cellular material was pooled and bath sonicated (Branson Instrument Company) for 2 minutes. Protein concentration of the sonicated material was determined by the method of Lowry (30) using bovine serum albumin as a standard.

Extracted nucleotides were separated on a 4.6 mm (I.D.) x 30 cm Partisil 10-SAX (Whatman) anion exchange column using a gradient of Buffer A (5 mM NH₃PO₄, pH 3.5) and Buffer B (500 mM NH₃PO₄, pH 4.8) at a flow rate of 2.0 ml/minute. The gradient was: 5 minutes 0% B, linear gradient at 4% B/minute, to 100% B. A Perkin Elmer dual pump Series 2 HPLC with a Perkin Elmer LC-75 detector was used. Absorbance was monitored at 254 nm and peak areas were determined with a Perkin Elmer Sigma 10 Data System. Standard solutions of nucleotides whose concentration had been established by U.V. absorbance were used to convert peak areas to nanomoles. Nucleotides from which standard nucleotide solutions were prepared were all purchased from the Sigma Chemical Company.

Results

Figures 22 and 23 show HPLC elution profiles of nucleotides extracted from equivalent numbers of V-79A and KC62 cells, respectively. The peak areas represent the total absorbance at 254 nm and are directly related to the total quantity of a given nucleotide in the extract sample. The elution profile for the V-79A extract (Figure 22) is almost identical to that for the KC62 extract (Figure 23). The same absorbance peaks are present in both elution profiles and the respective peak areas (as determined by integration) in the two elution profiles are very similar in most cases.

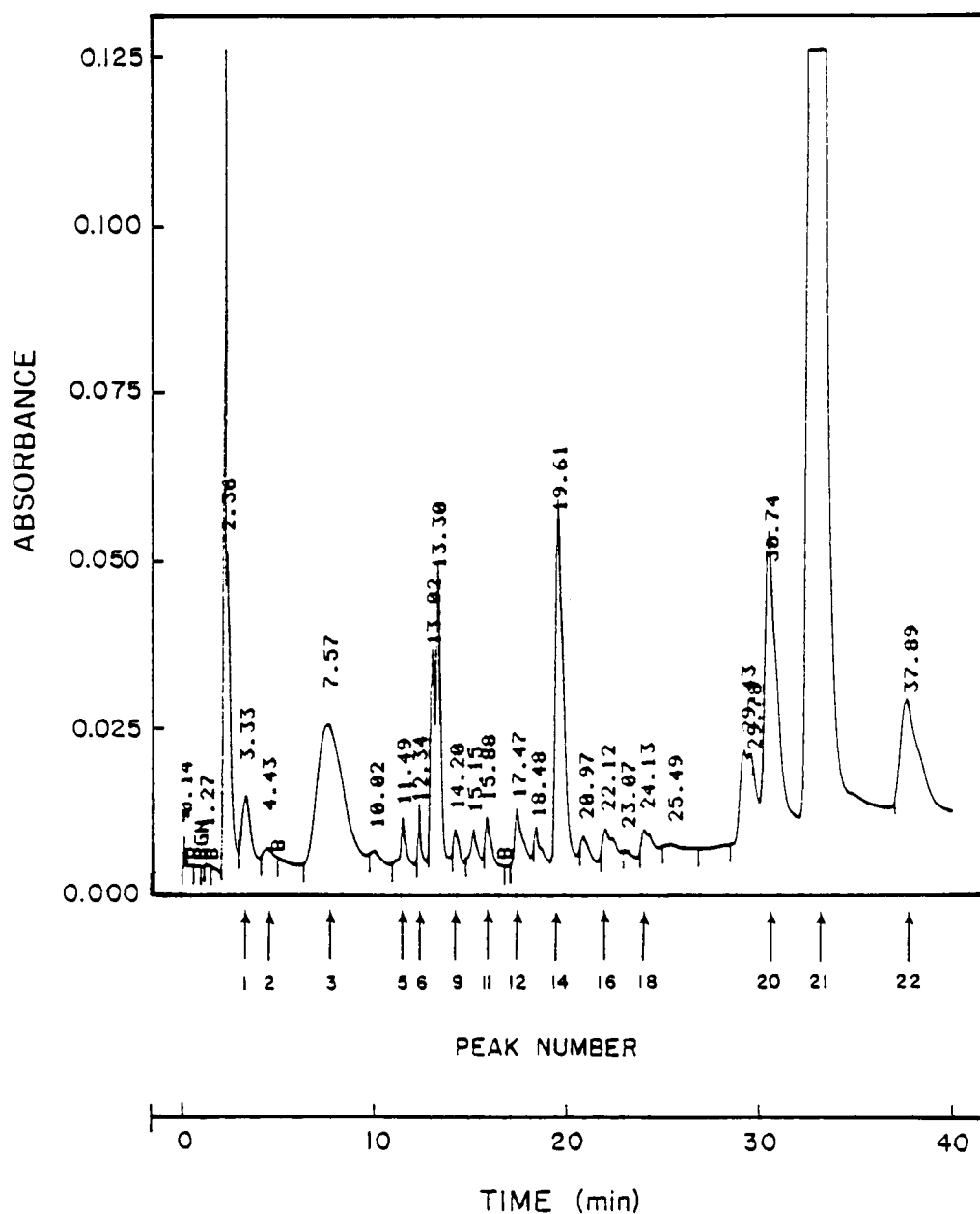


Figure 22. HPLC Chromatography of PCA-Soluble Nucleotides of V-79A Cells.

HPLC chromatography of a 150 μ l sample of PCA-soluble nucleotides extracted from a quantity of V-79A cells having a total protein content of 1.711 mg. Nucleotide elution from the column was monitored at 254 nm with a full scale deflection of 0.125 absorbance units.

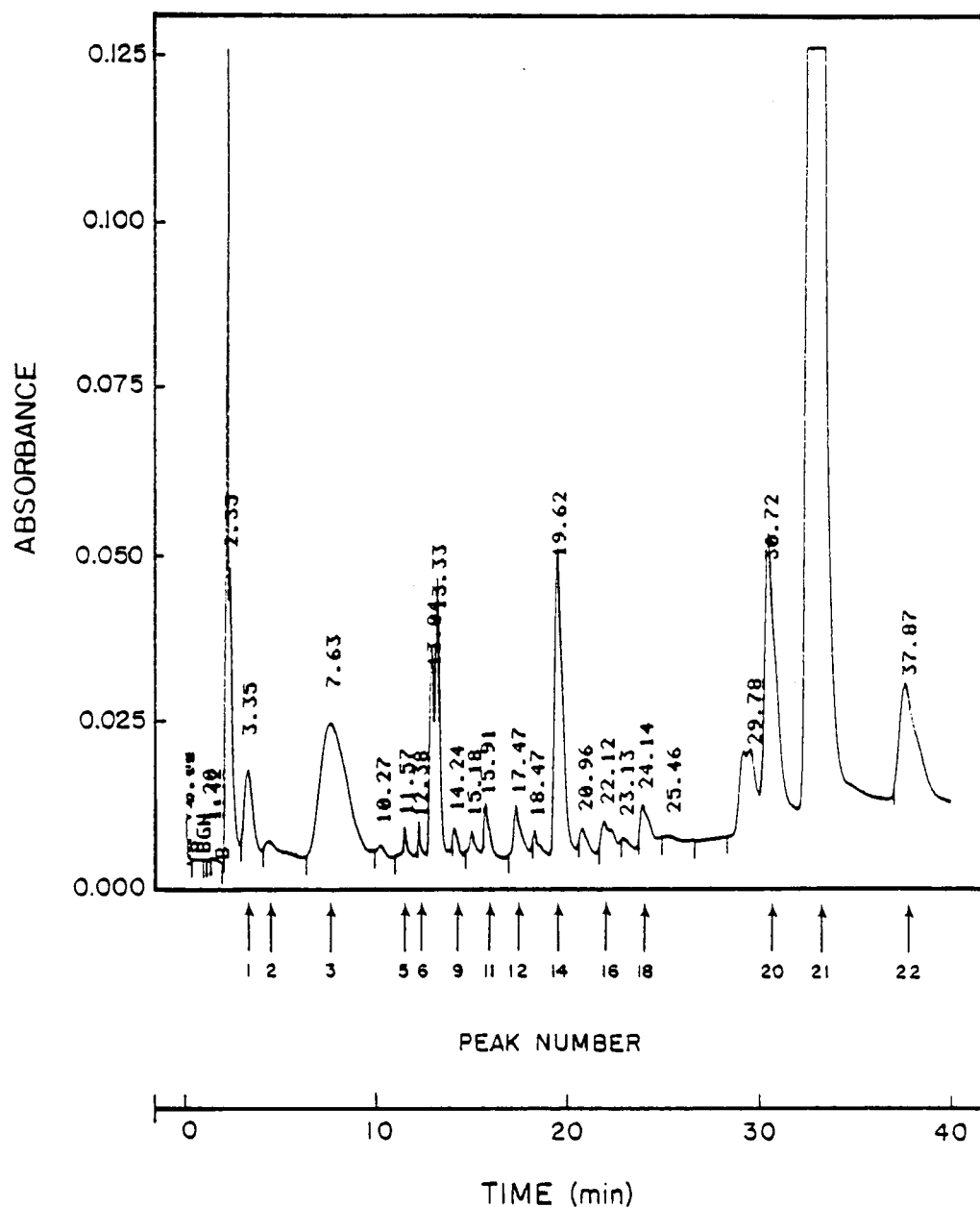


Figure 23. HPLC Chromatography of PCA-Soluble Nucleotides of KC62 Cells.

HPLC chromatography of a 150 μ l sample of PCA-soluble nucleotides extracted from a quantity of KC62 cells having a total protein content of 1.698 mg. Nucleotide elution from the column was monitored at 254 nm with a full scale deflection of 0.125 absorbance units.

The results of the nucleotide pool size determinations for V-79A and KC62 cells are summarized in Table VI. The values in Table VI represent average values from ten different extracts for each cell type. In most cases the nucleotide pools of the KC62 cells are approximately equal to those of V-79A cells. However, the IMP (Peak 5), GMP (Peak 6), NADH (Peak 9) and CDP (Peak 13) pools in KC62 cells appear to be significantly less than those of V-79A cells. The decrease in these particular nucleotide pools can be seen by comparison of the respective peak areas in Figures 22 and 23. The areas of Peak 18 in the elution profiles of KC62 extracts appears to be significantly larger than those for V-79A extracts. The identity of the nucleotide responsible for this peak is unknown.

Discussion

The procedure by which nucleotide extracts were prepared for HPLC has been commonly employed by numerous investigators (61,62,63). The KOH neutralization of PCA minimizes the breakdown of extracted nucleotides and prevents damage to the HPLC apparatus. The inclusion of a known quantity of ^{14}C -ATP in the PCA allowed monitoring of nucleotide dilution, breakdown, and loss in each step of the extraction procedure. Essentially no breakdown of ^{14}C -ATP was observed during the extraction procedure and loss of nucleotides was found to be less than 2%. In those cases where H^3 -hypoxanthine was substituted for ^{14}C -ATP in the PCA, nucleotide breakdown was not determined.

While the sizes of most of the nucleotide pools in the two cell lines are comparable, several nucleotide pools in the KC62 cells are significantly reduced. It has previously been shown that KC62's PRPP levels are approximately 30% of those of V-79A (27). It is reasonable to hypothesize that KC62's reduced PRPP levels are responsible for the alterations in its nucleotide pools. However,

TABLE VI.
NUCLEOTIDE POOLS IN V-79A AND KC62

Peak No.	Identity	V-79A ^a	KC62 ^a	62/79 x 100%
5	IMP	0.284 ± 0.023	0.150 ± 0.019	52.8%
6	GMP	0.223 ± 0.025	0.094 ± 0.019	42.2%
9	NADH	0.245 ± 0.020	0.168 ± 0.018	68.6%
12	UDP	0.480 ± 0.034	0.408 ± 0.029	85.0%
13	CDP	0.476 ± 0.029	0.294 ± 0.023	61.9%
14	ADP	1.890 ± 0.095	1.794 ± 0.103	94.9%
16	GDP	0.255 ± 0.037	0.221 ± 0.028	86.7%
19	CTP	2.550 ± 0.256	2.239 ± 0.153	99.4%
20	UTP	3.500 ± 0.399	3.410 ± 0.376	97.4%
21	ATP	20.500 ± 1.140	20.500 ± 1.120	100.0%
22	GTP	1.460 ± 0.248	1.510 ± 0.201	103.4%

Peak No.	Identity	V-79A ^b	KC62 ^b	62/79 x 100%
3	NAD + AMP	20.00 ± 1.050	19.60 ± 1.330	98.0%
7	UDP-sugar	4.56 ± 0.350	4.94 ± 0.381	108.0%
8	UDP-sugar	6.96 ± 0.438	6.73 ± 0.415	96.7%
1	?	3.64 ± 0.210	4.77 ± 0.381	131.0%
10	?	1.51 ± 0.116	1.21 ± 0.130	80.1%
11	?	1.53 ± 0.084	1.95 ± 0.110	127.5%
15	?	1.29 ± 0.090	1.23 ± 0.123	95.3%
18	?	1.39 ± 0.130	2.19 ± 0.157	157.8%

^aNanomoles/mg protein ± 1 S.D.

^bTotal absorbance/mg protein ± 1 S.D.

the specific mechanisms involved are not readily apparent. The available data make it possible only to speculate on the potential mechanisms by which reduced PRPP levels might result in the alterations present in the nucleotide pools of KC62.

In vivo, IMP production occurs primarily by means of the purine de novo and salvage pathways. Several investigators have demonstrated that reduced levels of PRPP can result in a decreased rate of de novo production of IMP (63). The production of IMP via phosphoribosylation of hypoxanthine is also contingent on the availability of PRPP since this salvage reaction is accomplished by the PRPP-utilizing enzyme hypoxanthine phosphoribosyltransferase. It has previously been shown that KC62 cells have a decreased capacity to salvage exogenously supplied hypoxanthine (27). The salvage of endogenously produced hypoxanthine might also be impaired by KC62's reduced PRPP levels. It is, therefore, possible that KC62's reduced IMP pool is a result of decreased IMP production by the purine de novo and/or salvage pathways.

The GMP pool of KC62 cells is also significantly reduced. As with IMP, GMP production occurs primarily by means of the purine de novo and salvage pathways. It has previously been shown that KC62 cells have a decreased capacity to salvage exogenously supplied guanine via the PRPP-utilizing phosphoribosylation reaction (27). The salvage of endogenously produced guanine might also be impaired by KC62's reduced PRPP levels leading to reduced GMP production and pool size. Operation of both the AMP and GMP arm of the de novo purine pathway are contingent on the availability of IMP. Usually a larger portion of the small IMP pool is diverted to the AMP arm than to the GMP arm suggesting that the regulatory mechanisms responsible are poised

in such a manner as to favor AMP production (64). If the IMP pool size is reduced, as is the case of KC62 cells, the AMP arm may be able to compete more effectively for available IMP. This could lead to decreased flux of IMP through the GMP arm and reduction in guanine nucleotide pools. In KC62 cells the GMP pool is significantly reduced while those of GDP and GTP are not. The mechanism(s) responsible for maintaining normal sized GDP and GTP pools in the presence of a decreased GMP pool is unclear. Generally, the relative amounts of mono-, di-, and triphosphates of all nucleotide families reflect the relative amounts of adenine nucleotides in each of the three levels of phosphorylation (65). It is possible that GDP and GTP pools are maintained by virtue of KC62's normal adenine nucleotide pools.

The mechanisms responsible for KC62's reduced CDP and NADH pools are unknown, but are probably related to KC62's reduced PRPP levels. Their reduction could be a direct result of KC62's reduced PRPP levels since synthesis of both compounds requires PRPP. It is also possible that their reduction is a secondary manifestation of the other alterations in KC62's nucleotide pools.

In only one other case have the nucleotide pools in cells with altered PRPP levels been examined. Valentine et al. (36,37,38) have examined erythrocytes from an individual suffering from nonspherocytic hemolytic anemia. Erythrocyte PRPP-synthetase activity was found to be 40 to 60% of normal and the AMP, ADP and ATP pools were 1.5 to 2.0 times normal. The investigators provided no explanation for the enlarged adenine nucleotide pools nor were the sizes of any other nucleotide pools determined.

CHAPTER VI

SUMMARY OF RESULTS

A previous investigation of the clonal cell line KC62 had shown it to have significantly reduced intracellular PRPP levels accompanied by a reduced ability to salvage exogenously supplied purines by phosphoribosylation when compared to the parental V-79A cell line. In an attempt to explain this phenotypic difference in the two cell lines, the physical and kinetic properties of the enzyme responsible for PRPP production, PRPP-synthetase, have been examined. Initial examination of the catalytic properties of the V-79A and KC62 PRPP-synthetase was conducted using 110,000 x G supernatant preparations of each enzyme. Determination of the kinetic parameters (K_m and V_{max}) for the V-79A and KC62 enzyme provided no evidence for a difference in their catalytic properties. Subsequent studies of the enzymatic activity in the presence of various nucleotides, however, demonstrated the KC62 enzyme to have an increased sensitivity to a variety of nucleotide inhibitors compared to its V-79A counterpart.

Prior to undertaking more extensive studies of the V-79A and KC62 PRPP-synthetases, a scheme for the purification of PRPP-synthetase was devised. This scheme permitted achievement of significant purification and reasonable recovery of activity while utilizing only a small amount of starting material.

The kinetic and physical properties of the purified V-79A and KC62 PRPP-synthetases have been examined. K_m values for the V-79A and KC62 PRPP-synthetases were found to be similar to each other as well as those previously obtained with 110,000 x G enzyme preparations. Inhibition of both enzymes by

AMP, GMP and XMP has been examined with respect to both substrates. For any given inhibitor and variable substrate, the resulting type of inhibition was found to be the same for the V-79A and KC62 enzyme. The apparent K_i values for the KC62 enzyme, however, were in all cases less than those of the V-79A enzyme. In several cases the K_i values for the KC62 enzyme were found to be four to five fold less than those of the V-79A enzyme. These results suggested that the KC62 enzyme possessed a structural alteration rendering it more sensitive to inhibition by nucleotide inhibitors. Examination of the physical properties of the two enzymes failed to provide evidence of a major structural difference between the two enzymes. KC62's subunit molecular weight, molecular weight, and pattern of thermal inactivation at 55°C was identical to that of the V-79A enzyme.

Finally, the consequences of KC62's reduced PRPP levels on its nucleotide pool sizes have been assessed. The IMP, GMP, NADH and CDP pools of the KC62 cells were significantly less than those of V-79A cells. While it is reasonable to attribute KC62's reduced nucleotide pools to its reduced PRPP levels, the specific mechanisms involved have not been determined.

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