Purification and Properties of 5-Phosphoribosyl Pyrophosphate Amidotransferase from Adenocarcinoma 755 Cells*

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ABSTRACT: 5-Phosphoribosyl 1-pyrophosphate amidotransferase has been partially purified from cultured Adenocarcinoma 755 cells by heat treatment, acid precipitation, and ammonium sulfate fractionation. Substrate saturation curves for 5-phosphoribosyl 1-pyrophosphate are sigmoid, and analysis of the data by the Hill equation shows an interaction coefficient of 1.9. The substrate concentration at half-maximal velocity, $S_{0.5}$, is 0.47 mM. Saturation curves for the other substrate, L-glutamine, show no interaction for sites which bind this compound; but increasing amounts of 5-phosphoribosyl 1-pyrophosphate increase the binding of L-glutamine to the enzyme. 6-Diazo-5-oxo-L-norleucine is a competitive inhibitor of the binding of L-glutamine, with an inhibition constant, K_i, of 12 μM. 2-Ethylamino-1,3,4-thiadiazole, a compound which apparently increases the rate of biosynthesis of nucleotides, does not stimulate 5-phosphoribosyl 1-pyrophosphate amidotransferase. All nucleotides tested inhibit the reaction to some extent. The most potent of all is 6-methylthiopurine ribonucleotide, for which a concentration of 90 μM is required for 50% inhibition of the reaction, $I_{0.5}$. Deoxyguanosine diphosphate, 6-thioguanylic acid, and 6-mercaptopurine ribonucleotide are also strong inhibitors; but inhibition by ribo- and deoxyribonucleoside triphosphates, except guanosine triphosphate, can be overcome at high magnesium ion concentration. Deoxyguanosine diphosphate and 6-thioguanylic acid display cooperativity between inhibitor binding sites, but the ribonucleotides of 6-mercaptopurine and 6-methylthiopurine do not.

The presence of the nucleotide inhibitors does not alter the $S_{0.5}$ value for 5-phosphoribosyl 1-pyrophosphate, and increased amounts of 5-phosphoribosyl 1-pyrophosphate do not alter the $I_{0.5}$ values for the inhibitors.

A control mechanism for purine biosynthesis involves feedback inhibition of PP-ribose-P[1] amidotransferase (ribosylamine 5-phosphate:pyrophosphate phosphoribosyltransferase (glutamate amidating), EC 2.4.2.14) by nucleotides. The reaction catalyzed by the enzyme is as follows

$$\text{L-glutamine} + \text{PP-ribose-P} + H_2O \xrightarrow{Mg^{2+}} \beta\text{-D-ribosylamine 5-phosphate} + \text{L-glutamate} + PP_i$$

PP-Ribose-P amidotransferase from avian liver has been purified and studied (Caskey *et al.*, 1964; Hartman, 1963a). This enzyme and that of bacteria (Rottman and Guarino, 1964; Nierlich and Magasanik, 1965) are inhibited by various ribonucleoside mono-, di-, and triphosphates. But the enzyme from mammalian sources has been only briefly characterized (Caskey *et al.*, 1964; Wyngaarden *et al.*, 1958), and studies with tumor cells have been limited to indirect assays which involve the use of purine bases to prevent the accumulation of 5′-phosphoribosyl-*N*-formylglycinamide in cells exposed to azaserine (LePage and Jones, 1961; Henderson, 1963; Bennett and Smithers, 1964; Brockman and Chumley, 1965).

Our efforts have been directed toward purification, study of the kinetic properties, and inhibition of PP-ribose-P amidotransferase from a mouse tumor, Adenocarcinoma 755, maintained in cell culture.

Experimental Procedures

The following compounds were prepared by the Organic Chemistry Division of Southern Research Institute: 6-mercaptopurine ribonucleotide (Montgomery and Thomas, 1961), 6-methylthiopurine ribonucleotide (Thomas and Montgomery, 1968), 6-thio-GMP (Roy *et al.*, 1961), 2-fluoro-AMP (J. A. Montgomery and K. Hewson, 1967, unpublished procedure), 6 mercaptopurine ribonucleoside 5′-*p*-nitrophenylphosphate (Thomas and Montgomery, 1968), 3′,5′-cyclic 6-mercaptopurine ribonucleotide (Thomas and Montgomery, 1968), 5-fluorodeoxyuridylyl-(5′→5′)-6-mercaptopurine ribonucleoside (Thomas and Montgomery, 1968), 6-azauridylyl-(5′→5′)-6-azauridine (Montgomery and and Thomas, 1967), bisthioinosine 5′,5‴-phosphate (Thomas and Montgomery, 1962), 2′-deoxy-5-iodouri-

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[1] The abbreviations used are: PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; 6-thio-GMP, 6-thioguanylic acid; 2-fluoro-AMP, 2-fluoroadenylic acid; 5-fluoro-UMP, 5-fluorouridylic acid; 6-aza-UMP, 6-azauridylic acid; 8-aza-GMP, 8-azaguanylic acid; azaserine, *O*-diazoacetyl-L-serine.

dylyl-(5′→5′)-2′-deoxyiodouridine (Montgomery and Thomas, 1967), and 2′-deoxy-5-bromouridylyl-(5′→5)-2′-deoxybromouridine (Montgomery and Thomas, 1967). 2-Ethylamino-1,3,4-thiadiazole was obtained from the Cancer Chemotherapy National Service Center. 5-Fluoro-UMP and 6-aza-UMP were obtained from P-L Biochemicals, Inc., through the Cancer Chemotherapy National Service Center. A sample of tubercidin 5′-phosphate (7-deazaadenosine 5′-phosphate) was kindly provided by Dr. A. Bloch, Roswell Park Memorial Institute, Buffalo, N. Y. Azaserine and 6-diazo-5-oxo-L-norleucine were obtained from Parke Davis and Co., Detroit, Mich., through the courtesy of Dr. John Dice. Nucleotides and the dimagnesium form of PP-ribose-P were purchased from P-L Biochemicals, Inc., and $(NH_4)_2SO_4$, special enzyme grade, from Mann Research Laboratories. Uniformly labeled L-[^{14}C]-glutamine was purchased from Schwartz BioResearch, Inc.

Adenocarcinoma 755 cells, grown in swirl cultures as described previously (Kelley *et al.*, 1961), were harvested by centrifugation, washed twice in 0.9% NaCl, and stored for 2–3 days at −10°.

The assay of Flaks and Lukens (1963) was used during purification of the enzyme. This assay involved incubation of the enzyme in the presence of PP-ribose-P, $MgCl_2$, NaF, Tris-Cl buffer (pH 7.5), and L-glutamine. The PP_i produced was precipitated as the manganese salt, and the washed precipitate was hydrolyzed and estimated colorimetrically as P_i.

The enzyme preparation obtained by the purification procedure was free of interfering enzymes, and the conversion of L-[^{14}C]glutamine into L-[^{14}C]glutamate was used as a simple assay procedure for all experiments except the purification steps. The standard assay consisted of 3.33 mM L-[^{14}C]glutamine, 42 mM Tris-maleate (pH 7.5), 1 mM PP-ribose-P, 2 mM magnesium ion, and enzyme in a final volume of 150 μl. The reaction was initiated by adding enzyme to the other components and was stopped by boiling for 1 min. Stopping the reaction by addition of EDTA was equivalent to the boiling step. All assays were made in duplicate. Incubation was for 20 min at 25°, unless otherwise stated.

Portions of the reaction mixture were streaked on strips of Whatman No. 3MM paper, which were subjected to chromatography for 16 hr in a solvent containing ethanol–*t*-butyl alcohol–formic acid–water (12:4:1:3, v/v). The R_F values for glutamine and glutamate are 0.35 and 0.55, respectively. The paper strips were scanned with a Packard 7201 radiochromatogram scanner equipped with an integrator to determine the extent of glutamate production.

A unit of enzyme activity is defined as that amount of enzyme producing 1 μmole of product in 20 min. Protein was determined by the method of Lowry *et al.* (1951).

Results

Purification of PP-ribose-P Amidotransferase. Stored Adenocarcinoma 755 cells (15–20 g wet weight) were thawed and broken in a homogenizer of the Potter-Elvejhem type. The broken cell preparation was centrifuged at 100,000*g* for 1 hr, and the supernatant was heated at 60° for 10 min. All subsequent operations were at 4°. After the coagulated protein was removed by centrifuging at 22,000*g*, the supernatant was adjusted to pH 5 with 1 M acetic acid and stirred for 10 min. The precipitate was collected by centrifuging at 22,000*g* and suspended in 10 ml of 25 mM Tris-maleate buffer (pH 7.5). $(NH_4)_2SO_4$ was added, and the protein precipitating between 40 and 60% of saturation was retained. This fraction was suspended in 1 ml of water and dialyzed for 90 min against 2 mM Tris-maleate (pH 7.5).

TABLE I: Purification of PP-ribose-P Amidotransferase.

Fraction	Enzyme Act. (units)	Protein (mg)	Units/mg
100,000*g* supernatant	7.0	522	0.013
Heat at 60°, 10 min	13.4	135	0.099
Precipitation at pH 5	30.7	53.2	0.577
$(NH_4)_2SO_4$ (0–40%)	3.95	34.2	0.115
$(NH_4)_2SO_4$ (40–60%)	7.84	6.45	1.21
$(NH_4)_2SO_4$ (60–90%)	<0.1	9.8	<0.01

A summary of a typical purification is found in Table I. The initial enzyme activity was very low, but the first steps of the purification resulted in an increase of total activity. The activity doubled on heating at 60° and doubled again on precipitation of the enzyme at pH 5. This may have been the result of removal of endogenous inhibitors. In the case cited, the specific activity increased 93-fold, but due to the increase in total activity, this represents only a 20-fold purification. Such purification was usually attained.

General. The enzyme preparation was unstable, as was the case for the pigeon liver enzyme (Wyngaarden and Ashton, 1959). Most of the activity was lost after 6 hr at 4°, and only half the activity could be retained by freezing overnight. Since attempts to stabilize the enzyme were not successful, we used it immediately after dialysis. Thus, the enzyme was prepared and used on the same day. Although the PP-ribose-P amidotransferase was labile, none of our preparations had lost sensitivity to nucleotide inhibitors. Such losses were a problem in the purification of the enzyme from pigeon liver (Caskey *et al.*, 1964). The enzymatic reaction was linear with time over the period assayed, and the rate of reaction was proportional to the amount of enzyme added. The preparation was free of glutaminase and ATPase activity. Omission of magnesium ion resulted in the loss of activity, as did the addition of EDTA.

pH Optimum. We investigated the effect of pH on en-

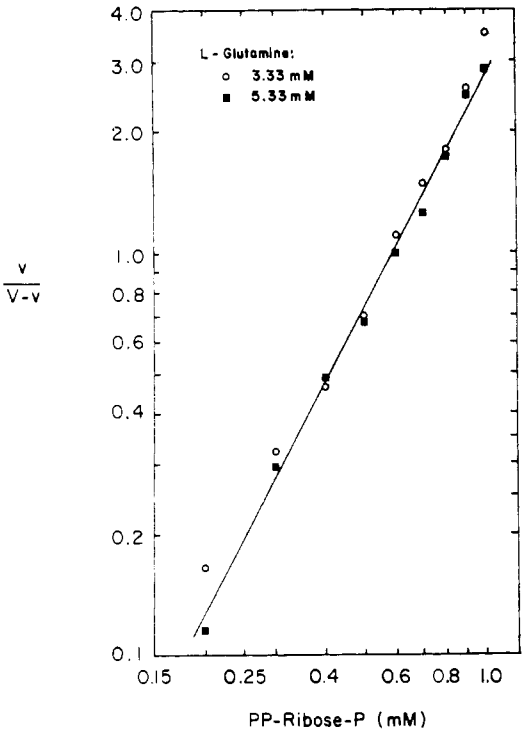

FIGURE 1: Cooperative binding of PP-ribose-P. Each assay contained the specified amount of PP-ribose-P and L-[^{14}C]glutamine. Otherwise the conditions were those of the standard assay. Enzyme (100 μg of protein) was added. With 1 mM PP-ribose-P and 3.33 mM L-glutamine, 156 mμmoles of glutamate was produced; with 5.33 mM L-glutamine, 196 mμmoles was formed.

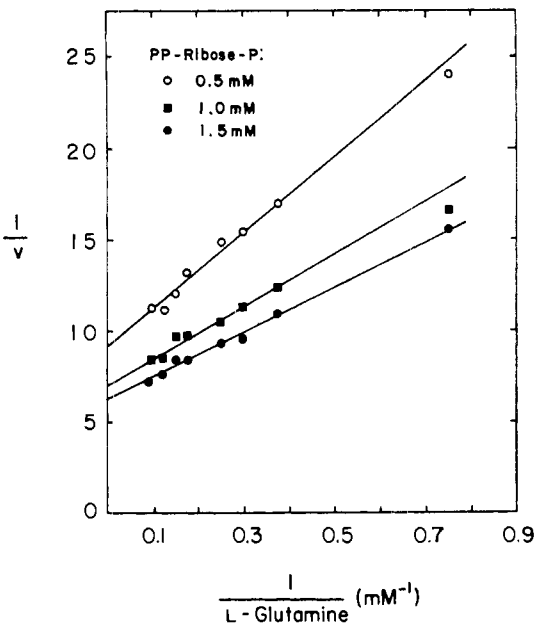

FIGURE 2: Effect of PP-ribose-P on L-glutamine binding. Conditions for assay were standard, except that the specified amounts of dimagnesium PP-ribose-P and L-glutamine were present and that enzyme (180 μg of protein) was added. Values for the ordinate are reciprocals of the micromoles of glutamate produced.

zyme activity. The optimum pH was 7.5 using Tris-maleate buffer and 7.0 with phosphate buffer. Sodium phosphate was a weak inhibitor of the reaction. At pH 7.0 the activity in the presence of phosphate buffer was 75% of that in the presence of an equivalent amount of Tris maleate; at pH 7.5 this value was 65%.

Binding of Substrates. We determined the apparent order of the reaction with respect to PP-ribose-P by use of the equation proposed by Hill (1910) to describe the kinetics of hemoglobin oxygenation (Figure 1). The equation as applied to PP-ribose-P amidotransferase is $\log v/(V - v) = \log K + n \log$[PP-ribose P], where v = velocity, V = maximum velocity, n = order of reaction or interaction coefficient, and K = apparent overall association constant. A plot of $\log v/(V - v)$ *vs.* log [PP-ribose-P] for eight separate experiments gave straight lines for which the slope, n, was 1.9 ± 0.2 (std dev). Similar data for other enzymes have been interpreted to mean that there is more than one binding site for substrate on the enzyme and that there is cooperativity between the sites, the value for n being a measure of the interaction (Atkinson *et al.*, 1965a,b; Monod *et al.*, 1965). The substrate concentration at half-maximum velocity ($S_{0.5}$) can also be determined from these plots. This value for PP-ribose-P from the eight experiments was 0.47 ± 0.07 mM (std dev). The addition of more L-glutamine increased the maximum velocity but did not change the $S_{0.5}$ value or the cooperativity for PP-ribose-P (Figure 1).

When the concentration of L-glutamine was varied while maintaining PP-ribose-P at constant levels, double-reciprocal plots of the Lineweaver–Burk type gave straight lines, indicating that there was no cooperativity between sites binding L-glutamine (Figure 2). However, with increasing amounts of PP-ribose-P, the K_m for L-glutamine decreased. At saturating levels of PP-ribose-P the K_m was 1.8 mM.

Inhibition by 6-Diazo-5-oxo-L-norleucine, Buffer, $(NH_4)_2SO_4$, and Metal Ions. The glutamine analog 6-diazo-5-oxo-L-norleucine was a potent competitive inhibitor of L-glutamine (Figure 3); the inhibition constant, K_i, was 12 μM. In contrast to the effect of 6-diazo-5-oxo-L-norleucine, azaserine, another glutamine analog, showed no inhibitory effect at concentrations up to 10 mM. Increasing the amount of Tris-maleate buffer inhibited the reaction slightly. Under standard assay conditions with 31 mM Tris-maleate, 228 mμmoles of glutamate was produced, and with 93 mM buffer 208 mμ moles was formed. A double-reciprocal plot showed the inhibition could not be overcome by increasing the amount of PP-ribose-P. $(NH_4)_2SO_4$ was a potent inhibitor, but its effect could be eliminated by increasing the PP-ribose-P concentration (Figure 4). Although the inhibition was competitive with PP-ribose-P, both the ammonium and the sulfate ions were involved in the inhibition. Relative rates in the presence of 1 mM PP-ribose-P were: no addition, 100; 33 mM $(NH_4)_2SO_4$, 48; 33 mM Na_2SO_4, 70; 67 mM NH_4Cl 84; and 67 mM NaCl, 99.

A number of divalent metal ions inhibited the reaction (Table II). Cupric, mercuric, cadmium, and zinc

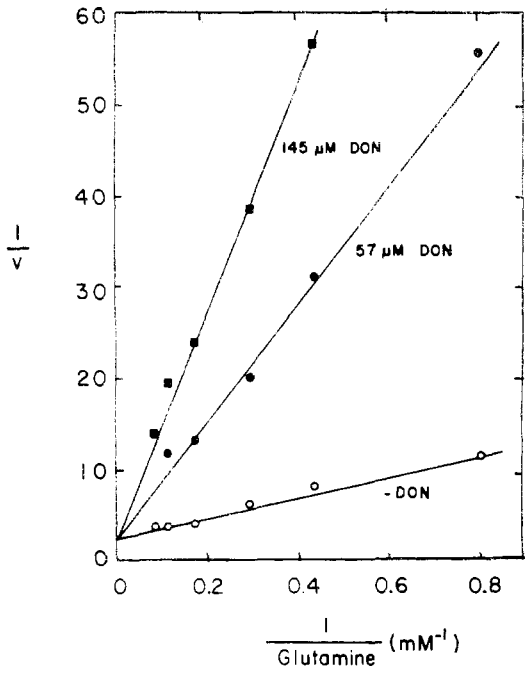

FIGURE 3: Inhibition of PP-ribose-P amidotransferase by 6-diazo-5-oxo-L-norleucine. Each assay contained 0.8 mM dimagnesium-PP-ribose-P, 36 mM Tris-maleate (pH 7.5), and the specified amount of L-glutamine and 6-diazo-5-oxo-L-norleucine in a total volume of 350 μl. Enzyme (150 μg of protein) was present, and incubation was for 30 min.

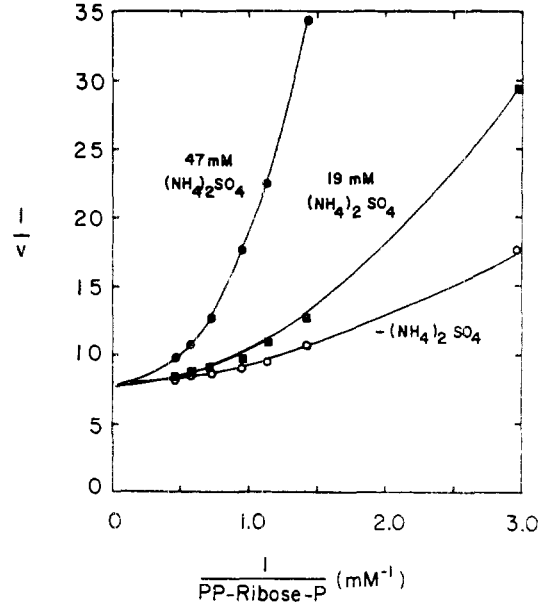

FIGURE 4: Inhibition of PP-ribose-P amidotransferase by $(NH_4)_2SO_4$. Standard conditions of assay were used, except that the specified amounts of PP-ribose-P and $(NH_4)_2SO_4$ were present. Enzyme (140 μg of protein) was added.

ions at concentrations of 0.167 mM had a strong effect, and at 16.7 mM nickel, calcium, cobalt, and manganese ions were inhibitory.

Assays in the Presence of 2-Ethylamino-1,3,4-thiadiazole. This agent is known to increase uric acid production (Krakoff and Balis, 1959). Two possible mechanisms for this action are stimulation of PP-ribose-P amidotransferase and reversal of the effect of feedback inhibitors of this enzyme. The compound did not stimulate the activity at either 0.2, 2.0, or 20.0 mM. In fact, the highest concentration inhibited 20%. Further, the inhibition produced by either AMP or 6-methylthiopurine ribonucleotide could not be reversed to a detectable degree by 2-ethylamino-1,3,4-thiadiazole at these concentrations. The lack of stimulation and lack of reversal of inhibition indicate that this enzyme is not a site of action of this compound.

Inhibition by Nucleotides and the Effect of Magnesium. Every nucleotide tested inhibited the reaction to some extent (Table III). However, the inhibition by ribo- and deoxyribonucleoside triphosphates could be overcome by increasing the magnesium ion concentration. GTP was a notable exception. Inhibition by the diphosphates was alleviated to a smaller extent, and that by the monophosphates was generally less affected by magnesium ions than the diphosphates. Apparently inhibition of enzyme activity by the triphosphates and, to a lesser extent, by the diphosphates and monophosphates in the presence of low magnesium ion concentration is due to removal of magnesium ion from the substrate, probably a dimagnesium-PP-ribose-P complex. In the absence of inhibitor, a ratio of 2:1 or greater for magnesium ion to PP-ribose-P concentration was optimal. At concentrations above the 2:1 ratio, magnesium ions had little effect on the reaction until they exceeded 10 mM. At levels higher than this, inhibition was evident.

Of those tested, the most potent, naturally occurring inhibitor was dGDP. The ribonucleotides of the purine analogs 6-mercaptopurine and 6-thioguanine were strong inhibitors, but the most potent of all was 6-methylthiopurine ribonucleotide. Inhibition by these four compounds is considered further in the final part of this section. Tests with several combinations of inhibitors revealed that no combination was more effective than an equivalent concentration of the more potent inhibitor. Strong synergistic effects for some combinations have been reported for studies with intact Ehrlich ascites cells (Henderson, 1963) and with the enzymes from pigeon liver (Caskey *et al.*, 1964) and *Aerobacter aerogenes* (Nierlich and Magasanik, 1965).

The following dinucleoside phosphates were tested for inhibition of PP-ribose-P amidotransferase:5-fluorodeoxyuridylyl-(5′→5′)-6-mercaptopurine ribonucleoside, 6-azauridylyl-(5′→5′)-6-azauridine, bisthioinosine 5′,5‴-phosphate, 2′-deoxy-5-iodouridylyl-(5′→5′)-2′-deoxyiodouridine, and 2′-deoxy-5-bromouridylyl-(5′→5′)-2′-deoxybromouridine. 6-Mercaptopurine ribonucleoside 5′-*p*-nitrophenyl phosphate and 3′,5′-cyclic 6-mercaptopurine ribonucleotide were also tested. Although some of these compounds were effective inhibitors, none was more potent than the nucleotide from which it was derived. We think that inhibition by these compounds is due to cleavage to the nucleotide by esterases present in the enzyme preparation. We observed the release of p-nitrophenol from 6-mercaptopurine ribonucleoside 5′-*p*-nitrophenylphosphate by the enzyme.

TABLE II: Inhibition of PP-ribose-P Amidotransferase by Metal Ions.[a]

Compd Tested	Rel Act. 16.7 mM	Rel Act. 0.167 mM
None	100	100
$Al_2(SO_4)_3$		97
$BaCl_2$	92	102
$CaCl_2$	25	91
$CdCl_2$		12
$CoCl_2$	23	102
$CuCl_2$		<3
$FeCl_3$		96
$FeSO_4$		102
$HgCl_2$		<3
KCl	90	99
LiCl	99	95
$MgCl_2$	73	98
$MnCl_2$	38	99
NaCl	97	100
$NiCl_2$	8	93
$PbCl_2$		50
$SnCl_2$		86
$ZnCl_2$		39

[a] Each assay contained 5 mM dimagnesium–PP-ribose-P, 125 mM Tris-maleate (pH 7.5), 1.33 mM L-[^{14}C]glutamine, the ion specified, and enzyme (143 μg of protein) in a final volume of 300 μl. Incubation was for 30 min at 25°. When no ion was added 132 mμmoles of glutamate was produced. At the higher concentration, precipitates were formed in some cases, preventing an adequate assay.

Effect of Inhibitors on the Binding of PP-ribose-P to the Enzyme. 6-Mercaptopurine ribonucleotide, 6-thio-GMP, and dGDP inhibited PP-ribose-P amidotransferase in a manner that was not reversed by increasing the PP-ribose-P concentration (Figure 5). A Hill plot of the data in Figure 5 showed that the cooperativity and the $S_{0.5}$ value for PP-ribose-P did not change in the presence of these inhibitors. Data on the inhibition by 6-methylthiopurine ribonucleotide were similar.

Effect of PP-ribose-P on the Binding of Inhibitors. Using the equation $\log v/(V_0 - v) = \log K' - n' \log I$, where v = velocity in the presence of inhibitor, V_0 = velocity in the absence of inhibitor, n' = apparent number of inhibitor molecules reacting with the enzyme to produce an inactive complex, I = inhibitor concentration, and K' = apparent over-all dissociation constant, one may determine if there is cooperativity between inhibitor binding sites (Taketa and Pogell, 1965). Plots of this type for 6-thio-GMP showed that there was cooperativity for this inhibitor. The n' value was greater than one (Figure 6). Four separate experiments with this analog gave a value of 1.6 ± 0.2 (standard deviation). Obtained from these plots, the amount required for $I_{0.5}$ was, with standard deviation, 0.27 ± 0.03 mM. The

TABLE III: Inhibition of PP-ribose-P Amidotransferase by Nucleotides and the Effect of Magnesium Ions on the Inhibition.[a]

Nucleotide	Concn (mM)	Rel Act. A	B	C	D
None		100	100	100	100
AMP	2.33	66	70	75	75
GMP	3.33	57	64	68	
IMP	6.67	55	45	62	
dAMP	6.67	53	61	59	
dGMP	3.33	50	53	66	
ADP	3.33	49	60	73	
GDP	3.33	44	58	64	
IDP	3.33	50	69	81	
dADP	3.33	66	82	85	
dGDP	1.11	48	43	66	76
ATP	2.00	60	91	94	
GTP	2.33	77	83	75	70
ITP	3.33	56	87	93	
dATP	1.67	86	103	105	
dGTP	3.33	54	90	98	
CTP	3.33	54	97	99	
UTP	3.33	60	92	92	
TTP	1.67	75	89	98	
dCTP	3.33	71	92	102	
dUTP	3.33	57	92	99	
5-Methyl-dCMP	3.33	45	55	62	
5-Fluoro-UMP	3.33	44	49	67	
2-Fluoro-AMP	1.67	45	57	68	64
6-Thio-GMP	0.23	53	54	57	64
8-Aza-GMP	3.33	39	51	59	
Tubercidin 5′-phosphate	2.33			75	
6-Mercaptopurine ribonucleotide	1.11	34	36	50	57
6-Methylthiopurine ribonucleotide	0.067	64	64	68	67

[a] The standard assay was used except that the specified amounts of magnesium ion and inhibitor were present. Enzyme (162 μg of protein) was added. The values are expressed as per cent of the glutamate produced in the absence of inhibitor and in the presence of the specified amount of magnesium. (A) 2 mM magnesium ion; (B) 2 mM magnesium ion plus additional magnesium ion equal to the concentration of the inhibitor; (C) 2 mM magnesium ion plus additional magnesium ion equal to twice the concentration of the inhibitor; and (D) 8.67 mM magnesium ion. The highest concentration of magnesium ion (15.3 mM) inhibited the reaction 20%. In the presence of 1 mM PP-ribose-P and 2 mM magnesium ion, 137 mμmoles of glutamate was produced. Other compounds, which were only slightly inhibitory, were: XMP, PP_i, DPN, TPN, DPNH, TPNH, CMP, UMP, TMP, CDP, dCMP, 2′- and 3′-AMP, 6-aza-UMP, TDP, 3′,5′-cyclic AMP, ribose 5-phosphate, and orotidylic acid. Free bases and ribonucleosides were not inhibitory.

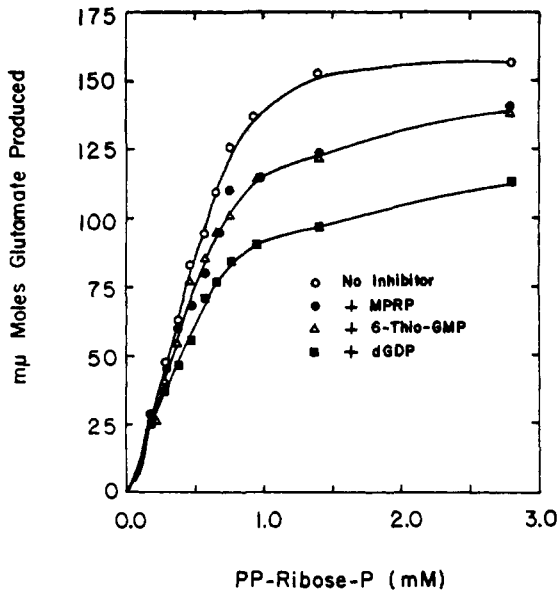

FIGURE 5: Effect of 6-mercaptopurine ribonucleotide, 6-thio-GMP, and dGDP on the binding of PP-ribose-P to the enzyme. Standard conditions of assay were followed, except that 6 mM $MgCl_2$ and enzyme (120 μg of protein) were present. In addition 6-mercaptopurine ribonucleotide (0.42 mM), 6-thio-GMP (0.11 mM), and dGDP (1.11 mM) were added as indicated. MPRP, 6-mercaptopurine ribonucleotide.

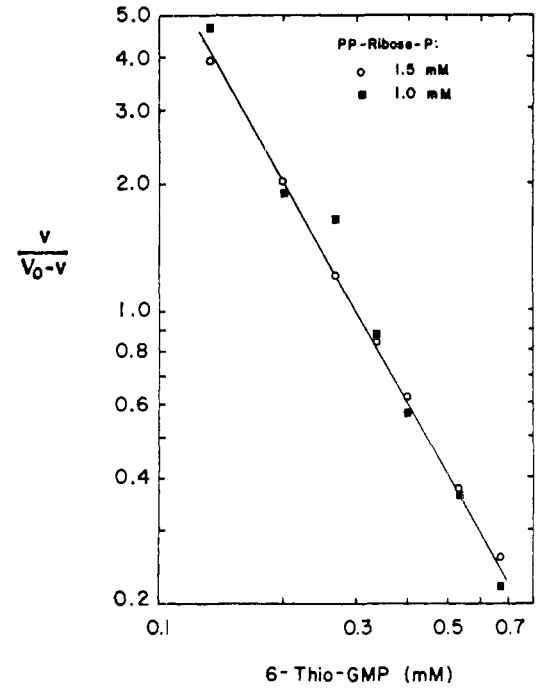

FIGURE 6: Effect of PP-ribose-P on inhibition by 6-thio-GMP. Assay conditions were standard, except that 8.67 mM magnesium ion, the indicated amounts of 6-thio-GMP, and enzyme (220 μg of protein) were present. With no inhibitor added, 156 and 118 mμmoles of glutamate were produced in the presence of 1.5 and 1.0 mM PP-ribose-P, respectively.

values for n' and $I_{0.5}$ were not changed by increasing the PP-ribose-P concentration. dGDP also had a high n' value. From four experiments similar to those for 6-thio-GMP, the value was 1.6 ± 0.2, and $I_{0.5}$ was 1.2 ± 0.4 mM. These values were also independent of PP-ribose-P concentration.

Figure 7 shows the results of increasing the amount of 6-methylthiopurine ribonucleotide at constant levels of PP-ribose-P. In the presence of either 0.5, 1.0, or 1.5 mM PP-ribose-P there was no evidence of a sigmoid curve. Also, the value for $I_{0.5}$ did not change with increasing PP-ribose-P concentration. Data from four separate experiments showed an n' value of 1.0 ± 0.1 and an $I_{0.5}$ value of 90 ± 15 μM. For 6-mercaptopurine ribonucleotide, the values from four experiments were $n' = 1.0 \pm 0.2$ and $I_{0.5} = 1.1 \pm 0.2$ mM. Sites on the enzyme for this inhibitor were also not cooperative, and increasing the PP-ribose-P concentration did not change the $I_{0.5}$ value.

In these experiments enough magnesium ion was added to eliminate the possibility of a competition between the nuçleotide and PP-ribose-P for this ion which would result in a lowering of the reaction velocity simply by removal of magnesium ion from the substrate.

Discussion

Striking differences between the PP-ribose-P amidotransferase of Adenocarcinoma 755 cells and those from pigeon liver (Wyngaarden and Ashton, 1959), rat liver (Caskey *et al.*, 1964), *Bacillus subtilis* (Rottman and Guarino, 1964), and *Aerobacter aerogenes* (Nierlich and Magasanik, 1965) are that the substrate saturation curve for the tumor cell enzyme is signoid and that inhibition by nucleotides is not overcome by PP-ribose-P. The other enzymes are reported to follow Michaelis–Menten kinetics, with the exception that, in the presence of inhibitor, the enzyme from *A. aerogenes* gives a sigmoid saturation curve (Nierlich and Magasanik, 1965). For these enzymes, the inhibition is competitive. A sigmoid saturation curve for the Adenocarcinoma 755 enzyme is consistent with the view that most enzymes subject to feedback inhibition do not follow Michaelis–Menten kinetics (Monod *et al.*, 1965).

The decreasing K_m for L-glutamine in the presence of increasing concentrations of PP-ribose-P indicates that PP-ribose-P binds to the enzyme before L-glutamine. This sequential binding of substrates by the enzyme from Adenocarcinoma 755 cells differs from that of pigeon liver, for which the binding is nonconsecutive at sites which are completely independent (Wyngaarden and Ashton, 1959). For the chicken liver enzyme, however, a combination of PP-ribose-P and magnesium promotes the covalent attachment of the glutamine analog 6-diazo-5-oxo-L-norleucine to the enzyme, indicating that the binding of PP-ribose-P and 6-diazo-5-oxo-L-norleucine is sequential. Presumably, the binding of PP-ribose-P and glutamine would also be sequential (Hartman, 1963b).

The effect of 6-diazo-5-oxo-L-norleucine and azaserine on the tumor cell enzyme is similar to that on enzymes from other sources. When there is no incubation of the

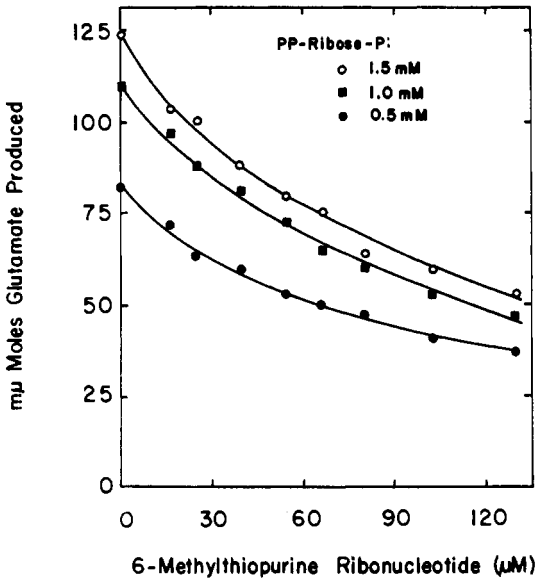

FIGURE 7: Effect of PP-ribose-P on inhibition by 6-methylthiopurine ribonucleotide. Standard conditions were followed, except that 8.67 mM magnesium ion, the indicated amounts of 6-methylthiopurine ribonucleotide, and enzyme (120 μg of protein) were present.

enzyme in the presence of 6-diazo-5-oxo-L-norleucine prior to assay, the inhibition is strictly competitive with L-glutamine (Hartman, 1963b). Azaserine does not inhibit the PP-ribose-P amidotransferase of Adenocarcinoma 755 cells, and it is also not an effective inhibitor of the enzyme of pigeon liver (Levenberg *et al.*, 1957), chicken liver (Hartman, 1963b), or mouse plasma cell neoplasm (Anderson and Brockman, 1963). Although 6-diazo-5-oxo-L-norleucine is a potent inhibitor of PP-ribose-P amidotransferase, its primary site of action on the purine pathway is at the conversion of 5′-phosphoribosyl-*N*-formylglycinamide into 5′-phosphoribosyl-*N*-formylglycinamidine (Anderson and Brockman, 1963; Tomisek and Reid, 1962). Azaserine also inhibits effectively at this step (Levenberg *et al.*, 1957).

The effect of magnesium ion on enzyme activity and inhibition is complex. A 2:1 ratio of magnesium ion to PP-ribose-P is required for optimal activity, and 2 and 1 mM concentrations of these were used in the standard assay. Ribonucleoside triphosphates have a strong affinity for magnesium (Walaas, 1958), and inhibition of PP-ribose-P amidotransferase by these compounds, except for GTP, is completely reversed by additional magnesium ion. At the relatively high concentrations of ribonucleoside triphosphates employed (1.67–3.33 mM), it seems likely that they inhibit through competition for magnesium with PP-ribose-P. High concentrations of magnesium ion have no effect on the inhibition of the pigeon liver enzyme by ATP (Wyngaarden and Ashton, 1959). For the enzyme of Adenocarcinoma 755, GTP is inhibitory at all concentrations of magnesium ion tested, from 2.0 to 8.67 mM. We conclude from this that both GTP and its magnesium complex are inhibitory. The ribonucleoside diphosphates bind magnesium ion less strongly than the triphosphates (Walaas, 1958), and their effect is only partly overcome by additional magnesium ion. Thus, they apparently inhibit only in part by competition for this ion. The ribonucleoside monophosphates bind magnesium ion even less strongly (Walaas, 1958), and magnesium ions have only a slight effect in reversing their inhibition.

PP-ribose-P amidotransferase of Adenocarcinoma 755 cells is generally not as sensitive to the naturally occurring purine ribonucleoside mono- and diphosphates as the PP-ribose-P amidotransferase of pigeon liver. Inhibition constants, K_i, for these compounds acting on the pigeon liver enzyme range from 30 μM to 5 mM (Caskey *et al.*, 1964). The sensitivity of the tumor cell enzyme to ribonucleotide inhibitors is greater, however, than that of chicken liver, for which a 1 mM concentration of AMP gave no inhibition (Hartman, 1963a).

The most potent, naturally occurring inhibitor of the tumor cell enzyme is dGDP; but it seems unlikely that this compound can accumulate in the cell to an extent that it becomes an effective inhibitor. Deoxyribonucleoside diphosphates cannot be detected in tissues of Novikoff hepatoma (Schneider, 1957). The monophosphates, diphosphates, and GTP are likely candidates for the role of feedback inhibitor inside the cell. Intact Adenocarcinoma 755 cells exposed to azaserine accumulate 5′-phosphoribosyl-*N*-formylglycinamide intracellularly, but the presence of either adenine or hypoxanthine in the medium greatly reduces the accumulation (Brockman and Chumley, 1965). Guanine is less effective, perhaps due to the high activity of guanase. The purine bases are effective only if they are converted into the nucleotide (Brockman and Chumley, 1965). Data similar to these have been obtained with Ehrlich ascites cells, although there is some question as to which purine bases are more potent (LePage and Jones, 1961; Henderson, 1962). It is evident that derivatives of some purine bases can function as feedback inhibitors in the intact tumor cell.

The inhibition of PP-ribose-P amidotransferase by 5-fluoro-UMP and 5-methyl-dCMP is interesting. Although these compounds are not potent inhibitors, they are the only pyrimidine derivatives found to inhibit without reversal by additional magnesium ion. This ion overcomes the inhibition by CTP, dCTP, UTP, dUTP, and TTP. 2-Fluoro-AMP is moderately effective as an inhibitor. From studies using the technique of blocking purine biosynthesis with azaserine, 2-fluoroadenine is known to be a feedback inhibitor in *Escherichia coli* and human epidermoid carcinoma cells in culture (Bennett and Smithers, 1964). 8-Aza-GMP is not a potent inhibitor of PP-ribose-P amidotransferase of Adenocarcinoma 755 cells. It also does not strongly inhibit the enzyme from pigeon liver (McCollister *et al.*, 1964). Further, 8-azaguanine does not have a strong effect on the accumulation of 5′-phosphoribosyl-*N*-formylglycinamide in azaserine-treated Adenocarcinoma 755 cells (Brockman and Chumley, 1965) or Ehrlich ascites cells (Henderson, 1963). The primary action of 8-azaguanine on cells is thought to occur by its incorporation into mRNA, with subsequent effects on protein synthesis (Chantrenne and Devreux, 1960; Grünberger *et al.*,

1966). Inhibition by tubercidin 5′-phosphate is no stronger than that by AMP.

6-Thioguanine and 6-mercaptopurine are purine antimetabolites that possess growth-inhibitory activity against both transplanted tumors in animals and neoplasms in man. The ribonucleotides of these compounds are good inhibitors of PP-ribose-P amidotransferase of Adenocarcinoma 755 cells, and both inhibit the accumulation of 5′-phorphoribosyl-*N*-formylglycinamide in these cells treated with azaserine (Brockman and Chumley, 1965). The ribonucleotides are inhibitors of the enzyme isolated from pigeon liver (McCollister *et al.*, 1964) and addition of the bases reduces the accumulation of 5′-phosphoribosyl-*N*-formylglycinamide in Ehrlich ascites cells treated with azaserine (LePage and Jones, 1961).

There is strong evidence that some metabolite of 6-mercaptopurine inhibits an early step in the purine biosynthetic pathway (presumably PP-ribose-P amidotransferase) as the primary site of its action in blocking the formation of purines (Bennett *et al.*, 1963; Hakala and Nichol, 1964). 6-Mercaptopurine ribonucleotide may be methylated by tumor cells to produce 6-methylthiopurine ribonucleotide (Allan *et al.*, 1966), the most potent inhibitor of all those examined for activity against PP-ribose-P amidotransferase. 6-Methylthiopurine ribonucleoside is also extremely effective in preventing 5′-phosphoribosyl-*N*-formylglycinamide accumulation in tumor cells treated with azaserine (Henderson, 1962; Bennett *et al.*, 1965). Thus, it appears that most of the effect of 6-mercaptopurine on the biosynthesis of purines is due to its conversion to 6-methylthiopurine ribonucleotide.

The allosteric properties of PP-ribose-P amidotransferase of Adenocarinoma 755 cells are interesting. The kinetics do not follow those specified by the model proposed by Monod *et al.* (1965). It cannot be classified with their "K" system, because the $S_{0.5}$ value for PP-ribose-P is not altered in the presence of inhibitor. Further, it does not belong to the "V" system, for this system requires that the substrate should not exhibit cooperative homotropic interactions. Our data show that PP-ribose-P has an n value of 1.9. Another requirement for the proposed model is that inhibitors should display cooperative homotropic interactions. Although this is the case for dGDP and 6-thio-GMP, two other inhibitors, the ribonucleotides of 6-mercaptopurine and 6-methylthiopurine, display no cooperativity. Several enzymes which do not follow the proposed model have been found (Atkinson *et al.*, 1965a,b; Preiss *et al.*, 1966), but the PP-ribose-P amidotransferase studied by us appears to be unique in that the V_{max} decreases in the presence of inhibitor while substrate saturation curves remain sigmoid.

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Role and Reactivity of Sulfhydryl Groups in Firefly Luciferase*

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ABSTRACT: The chloromethyl ketone derivative of *N*-tosyl-L-phenylalanine is an effective inhibitor of firefly luciferase. The inhibition of enzymatic activity is accompanied by a loss of approximately two sulfhydryl groups. The chloromethyl ketone derivative of *N*-tosyl-L-lysine is without effect under similar conditions. Kinetically, *N*-tosyl-L-phenylalanine chloromethyl ketone inhibition is competitive with respect to one of the substrates, luciferin, and noncompetitive with respect to the other, adenosine 5′-triphosphate. The hydrophobic character of *N*-tosyl-L-phenylalanine chloromethyl ketone appears to be the major factor for its binding to the active site of luciferase. The fact that *N*-tosyl-L-phenylalanine is a competitive inhibitor also with a K_i of the same order of magnitude as *N*-tosyl-L-phenylalanine chloromethyl ketone supports this conclusion. It appears, therefore, that *N*-tosyl-L-phenylalanine chloromethyl ketone inhibition of luciferase involves two phases: first, a reversible binding of *N*-tosyl-L-phenylalanine chloromethyl ketone molecules at the luciferin binding sites (demonstrable kinetically as competitive inhibition), and second, the reaction of the chloromethyl ketone group of *N*-tosyl-L-phenylalanine chloromethyl ketone with the SH groups at or near these sites. Inactivation by *N*-tosyl-L-phenylalanine chloromethyl ketone is pH dependent; inactivation–pH curve corresponds very closely to the luciferase activity–pH curve. It is suggested that two sulfhydryl groups are located at or near the binding site of luciferin, and that they are required in the luciferase-catalyzed production of yellow-green light.

Firefly luciferase catalyzes the following reactions (Rhodes and McElroy, 1958)

$$\text{enzyme} + \text{luciferin} + \text{ATP} \rightleftarrows \text{enzyme–luciferin–AMP} + \text{PP} \quad (1)$$

$$\text{enzyme–luciferin–AMP} + O_2 \longrightarrow \text{light} + \text{products} \quad (2)$$

$$\text{enzyme} + \text{dehydroluciferin} + \text{ATP} \rightleftarrows \text{enzyme–dehydroluciferin–AMP} + \text{PP} \quad (3)$$

DeLuca *et al.* (1964) have shown that two sulfhydryl groups on the enzyme are necessary for maintaining catalytic activity; the remaining six sulfhydryl groups could be modified without affecting enzymatic function. The amino acid sequence surrounding the two essential sulfhydryl groups has been determined (Travis and McElroy, 1966).

Since luciferase undergoes a large conformational change in the process of enzyme–dehydroluciferyl-adenylate complex formation (DeLuca and Marsh, 1967), it is not possible to determine whether the two essential sulfhydryl groups are at the catalytic site or are simply necessary for maintaining the structural changes that occur during the activation reaction.

Recently we found that TPCK,[1] an aromatic inhibitor of chymotrypsin, also inhibits luciferase activity. The results of these inhibition studies suggest that there are essential sulfhydryl groups present at the luciferin binding site.

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[1] Abbreviations used: TPCK, the chloromethyl ketone derivative of *N*-tosyl-L-phenylalanine; TLCK, the chloromethyl ketone derivative of *N*-tosyl-L-lysine.