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ADENOSINE PHOSPHORYLASE ACTIVITY AS DISTINCT FROM INOSINE-GUANOSINE PHOSPHORYLASE ACTIVITY IN SARCOMA 180 CELLS AND RAT LIVER

ASHOK Y. DIVEKAR*

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, 666 Elm Street, Buffalo, N.Y. 14263 (U.S.A.)

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Summary

Adenosine phosphorylase (EC 2.4.2.-) activity present in Sarcoma 180 cells grown in culture and in rat liver, is shown to be distinct from inosine-guanosine phosphorylase by several criteria: (a) treatment of Sarcoma 180 cell extract with *p*-chloromercuribenzoate inhibited the two activities to a different extent, (b) adenine selectively protected the adenosine phosphorylase activity of Sarcoma 180 and rat liver extract against heat inactivation, while hypoxanthine selectively protected inosine-guanosine phosphorylase activity, (c) at nearly saturating substrate concentrations and using Sarcoma 180 extract, the rates of ribosylation of a mixture of adenine + hypoxanthine or adenine + guanine, but not of hypoxanthine + guanine, were found to be almost equal to the sum of their individual rates as measured separately, (d) inosine selectively inhibited the ribosylation of hypoxanthine and guanine catalysed by Sarcoma 180 and rat liver extract while 2-chloroadenosine selectively inhibited the ribosylation of adenine and N^6-furfuryladenine, (e) pH vs. activity curves were similar with hypoxanthine or guanine as the substrate but they were markedly different from the curve with adenine as the substrate. The potential role of adenosine phosphorylase activity in vivo is discussed.

Introduction

There are three possible pathways by which adenosine can be metabolized in mammalian cells (Fig. 1). Phosphorylation by adenosine kinase and deamina-

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

* Present address: Department of Pharmacology, University of Puerto Rico, Medical Sciences Campus, G.P.O. Box 5067, San Juan, Puerto Rico 00936.

tion by adenosine deaminase occur in cell-free extracts of several mammalian tissues. Purine nucleoside phosphorylase (purine nucleoside, orthophosphate ribosyl transferase, EC 2.4.2.1) which catalyzes a reversible phosphorolysis of purine nucleosides, is generally believed to be inactive towards adenosine [1—3]. The study of the phosphorolysis of adenosine in crude cell extracts is difficult because of the abundance of adenosine deaminase, but the reverse reaction can be studied because of the absence of adenine deaminase in mammalian cells [2]. Zimmerman et al. [4] recently reported that crude extracts of several rat tissues as well as crystalline purine nucleoside phosphorylases isolated from calf spleen and human erythrocytes catalyzed a very slow ribosylation of adenine, the rate being 0.06 to 0.3% of that of hypoxanthine. Based on Bio-Gel P 150 elution profiles and competitive inhibition of selenoguanine ribosylation by adenine these investigators concluded that one enzyme catalyzed the ribosylation of adenine and hypoxanthine but questioned its physiological significance in the metabolism of adenosine in view of the relatively low reaction velocity and a 10-fold higher K_m for adenine as compared to hypoxanthine.

In view of the rapid conversion of adenosine and its metabolic products, adenine and inosine (see Fig. 1) to adenine nucleotides, it becomes difficult to assess the extent of adenosine phosphorolysis in vivo. Several indirect observations indicate the possibility that adenosine (and analogues) may undergo phosphorolysis in vivo. Thus, normal humans are known to excrete small quantities of adenine in the urine even when maintained on a purine-free diet [5]. Also, injection of [8-^{14}C]hypoxanthine or guanine in xanthurenics resulted in small but definite excretion of labeled adenine in the urine, in spite of the extremely efficient salvage of exogenously administered adenine [6]. High activity of adenine phosphoribosyl transferase is present in several mammalian tissues [2] and the activity in the liver increases after partial hepatectomy indicating thereby the possible role of this enzyme in the metabolism of rapidly growing cells. The substrate for this enzyme, adenine, could arise in the cells only via phosphorolysis of adenosine (Fig. 1). Since preformed adenine is not essential in mammalian nutrition [7], on teleological grounds, one would expect that suf-

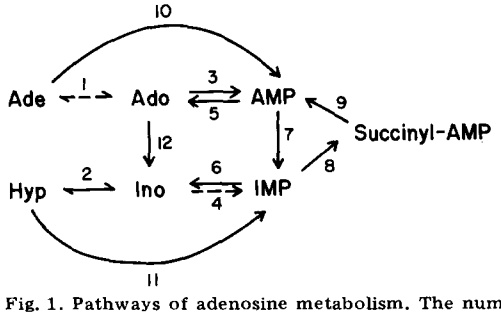

Fig. 1. Pathways of adenosine metabolism. The numbers designate the following enzymes: (1) Adenosine phosphorylase; (2) Inosine-guanosine phosphorylase; (3) Adenosine kinase; (4) Inosine kinase; (5,6) 5′-nucleotidase; (7) AMP deaminase; (8) Adenylosuccinate synthetase; (9) Adenylosuccinate lyase; (10) Adenine phosphoribosyl transferase and (11) Hypoxanthine-Guanine phosphoribosyl transferase; (12) Adenosine deaminase. Reactions indicated by dashed arrows are not thought to be physiologically significant in mammalian tissues.

ficient adenine is produced in the cells by catabolism of nucleic acids and nucleotides. It is of interest that the purine nucleoside phosphorylase purified from embryonic chicken liver was active towards adenosine (approximately 10% as compared to inosine) while the enzyme from adult chicken liver was inactive [8].

Recently, N^6-(Δ^2-isopentenyl)adenosine and N^6-benzyladenosine were shown to undergo rapid phosphorolysis in man [9,10] as well as in mammalian cells in culture [11,12]. The corresponding free bases are biologically inactive, presumably because unlike the nucleosides, they cannot be metabolized to active nucleotide forms [13]. Thus, the phosphorolytic cleavage apparently limits the therapeutic usefulness of these analogues in the treatment of cancer. In contrast, with another adenosine analogue, 2-fluoroadenosine, the principal route of toxic nucleotides formation appeared to be via initial conversion to 2-fluoroadenine [14,15]. Glycosidic cleavage of deoxyadenosine has also been shown to occur in several intact mammalian cells in the presence of coformycin, a potent inhibitor of adenosine deaminase [16].

In view of the physiological and pharmacological significance of the phosphorolytic cleavage of adenosine and analogues and the fact that purine metabolizing enzymes often show specificity toward the substituent at 6 position, we wished to determine whether the phosphorolysis of 6-amino and -oxopurines is catalysed by a single enzyme. The results reported in this communication suggest the existence of a purine nucleoside phosphorylase activity which is specific towards adenosine and distinct from inosine-guanosine phosphorylase, in Sarcoma 180 cells grown in vitro and in rat liver. An abstract on this subject has been published [17].

Materials and Methods

Compounds. The following were purchased from the sources indicated. Adenine sulphate, guanine hydrochloride, guanosine, hypoxanthine, inosine, N^6-furfuryladenine and *p*-chloromercuribenzoic acid (Na salt) from ICN Pharmaceuticals Inc., Cleveland, Ohio, U.S.A.; N^6-(Δ^2-isopentenyl)adenosine from Stark Associates, Buffalo, N.Y., U.S.A.; 2-chloroadenosine from Cyclo Chemicals, Los Angeles, Calif., U.S.A.; glycylglycine, imidazole, *N*2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), Tris and ribose-1-phosphate (dicyclohexylammonium salt) from Sigma Chemical Co., St. Louis, Mo., U.S.A. [8-^{14}C]labeled compounds, adenine (53 mCi/mmol), hypoxanthine (59 mCi/mmol), guanine (55 mCi/mmol) and N^6-furfuryladenine (17 mCi/mmol) from Amersham Searle Corp., Arlington Heights, Ill., U.S.A. [8-^{14}C]guanosine (34 mCi/mmol) from Schwartz Mann, Orangeburg, N.Y., U.S.A. N^6-(Δ^2-isopentenyl)-[8-^{14}C]adenosine (50 mCi/mmol) was synthesized and kindly supplied by Dr. M.H. Fleysher of this department [18]. On paper chromatography in ethanol/ammonium biborate (9 : 1, v/v) all radioactive compounds, with the exception of N^6-furfuryladenine, showed a single peak and were over 99% pure. N^6-furfuryladenine showed about 2% radioactive impurity, which moved on the chromatogram like N^6-furfuryladenosine. The specific activity of labeled compounds employed in all experiments except K_m determinations was adjusted to 1 μCi/μmol by addition of unlabeled compounds. For K_m determina-

tions solutions of specific activity 10 μCi/μmol were used. The solutions were stored frozen at −20°C.

Cells. Mouse Sarcoma 180 cells used in this study were grown in roller bottles, harvested and stored frozen at −70°C as described earlier [19]. They were used within 6 months. The cultures of cells were tested and found to be free of pleuro-pneumonia-like organisms [20].

Cell and tissue extracts. Extracts of Sarcoma 180 cells were prepared in 5 mM Tris, pH 7.4, by freezing and thawing three times. The suspension was centrifuged at 105 000 × *g* for 60 min, the supernatant was dialyzed for 16 h against the same buffer and recentrifuged at the same speed for 30 min [21]. The supernatant was used for assay and was stored frozen at −70°C. Under these conditions the enzymes assayed were stable for at least 2 weeks. Protein was determined by the method of Lowry et al. [22]. Rat liver extract was prepared by homogenizing finely cut liver of Sprague-Dawley female rats (200 g) in a Potter Elvjehm homogenizer (~30 up-down strokes) in 5 mM Tris, pH 7.4. Dialyzed 105 000 × *g* supernatant (see above) was used as the source of the enzyme.

Nucleoside phosphorylase assay. The assay was based on the conversion of [8-^{14}C] labeled purines to the corresponding nucleosides and their estimation after chromatographic separation from the remaining substrate. Unless otherwise specified, the reaction mixture (0.4 ml) contained: 125 μM [8-^{14}C]purine (250 μM of N^6-furfuryladenine), 1 mM ribose-1-phosphate, 63 mM glycylglycine buffer, pH 7.4 and the cell extract (8—1000 μg protein). The reaction was started by addition of the cell extract to the prewarmed assay mixture (35°). After incubation at 35°C (1—16 min) the reaction was stopped by addition of 20 μl of cold 60% trichloroacetic acid. The precipitate was removed by centrifugation and 50 μl aliquot was subjected to descending chromatography for 4 h at room temperature on Whatman 3 MM paper (2.5 cm × 40 cm) with 0.1 M ammonium biborate/ethanol (9 : 1, v/v) as the solvent. The chromatogram was cut into 1 cm sections and counted for ^{14}C in a Packard Tri-Carb liquid scintillation spectrometer (Model 2450) with a 65% counting efficiency [19]. Labeled sections were counted to obtain a minimum of 10 000 counts. In the mixed substrate experiments, inosine and guanosine were separated from the purine bases hypoxanthine and guanine by electrophoresis (Shandon high voltage electrophoresis unit, Model Q11) of a 50 μl aliquot of the assay mixture on Whatman 3MM paper strips, 2.5 cm × 25 cm, using 20 mM ammonium biborate, pH 8.9 (2500 V; 60 min). This procedure was also employed for separating xanthine and uric acid from hypoxanthine.

For studying the reverse reaction, namely the conversion of nucleosides to purines, the assay mixture (0.4 ml) contained: 125 μM [8-^{14}C]nucleoside, 63 mM potassium phosphate, pH 7.4, and the cell extract.

Results

R_F values and electrophoretic mobilities. Paper chromatography using ethanol/ammonium biborate as the solvent gave clearcut separation of each of the purine ribonucleoside from the corresponding free base in 4 h. At room temperature (18—25°C) approximate R_F values for the various compounds

were: adenine and guanine, 0.4; hypoxanthine, N^6-furfuryladenine and N^6-(Δ^2-isopentenyl)adenine, 0.6; inosine, N^6-furfuryladenosine and N^6-(Δ^2-isopentenyl)adenosine, 0.8; adenosine and guanosine, 0.7. The chromatographic procedure did not give good separation of guanosine, xanthine and uric acid from hypoxanthine. These separations were obtained by electrophoresis. On electrophoresis guanosine, inosine, xanthine and uric acid migrated towards the anode (9—14 cm) while hypoxanthine and guanine remained at the origin.

Nucleoside phosphorylase activity of Sarcoma 180 cell extract. The rate of ribonucleoside formation from purine bases, adenine, N^6-furfuryladenine, hypoxanthine and guanine or the phosphorolysis of N^6-(Δ^2-isopentenyl)adenosine and guanosine to purines was linear with respect to time (30 min) and protein till at least 35% of the substrate was exhausted (data not shown). The chromatogram of each assay mixture, except those containing adenine, showed only two radioactive peaks, one corresponding to the base and the other to the corresponding ribonucleoside. In the case of adenine assay, the chromatogram showed two major radioactive peaks, one corresponding to adenine, the other to inosine and a third small peak in the region of adenosine. The rate of adenine ribosylation was calculated from the sum of the counts appearing in inosine and adenosine, although generally the cpm in adenosine were less than 10% of those in inosine. Inosine was apparently formed from adenosine by the deaminase present in the crude cell extract. The specific activity of adenosine deaminase in the extract was about 10-fold higher than adenine ribosylating activity [23]. The identity of each of the nucleosides formed in all of the above experiments was established by chromatography in two solvent systems employing authentic compounds as internal markers. Preliminary experiments (not shown) established that the conversion of purine bases to nucleosides was dependent on the addition of ribose-1-phosphate, while the phosphorolysis of nucleosides required the presence of P_i (<10% conversion in the absence of ribose-1-phosphate or P_i). At pH 7.4 the ribosylation of adenine, but not of hypoxanthine, was significantly affected by the type of buffer used. Thus the rate in 63 mM glycylglycine or HEPES was approximately 1.5 times that observed in Tris or imidazole buffer of the same pH and molarity. The enzyme activity in glycylglycine buffer with adenine was 7.5—12.2 (average 9.9, 5 different extracts) nmol/mg protein/min. The activity with N^6-furfuryladenine was about the same, while the activity with hypoxanthine or guanine was 60—90 nmol/mg protein/min. At pH 7.4 the K_m values for adenine, N^6-furfuryladenine, hypoxanthine and guanine were 27, 67, 13 and 21 μM respectively (Fig. 2), while at pH 5.0 the values were 7, 50, 18 and 5 μM, respectively.

The K_m for hypoxanthine found in this study is comparable to the values reported by Zimmerman et al. [4] for crystalline enzyme from human erythrocytes and partially purified rat liver enzyme. Similarly the K_m for guanine is comparable to the K_i reported for the highly purified human erythrocytic enzyme [24]. However, markedly different K_m values for these substrates have been reported (for discussion, see ref. 3). The K_m for adenine in the case of the Sarcoma 180 enzyme is only about 1/10 of that reported for human erythrocyte or rat liver enzyme [4]. At pH 7.4 and with 125 μM hypoxanthine, the K_m for ribose-1-phosphate was 66 μM (data not shown) as compared to about

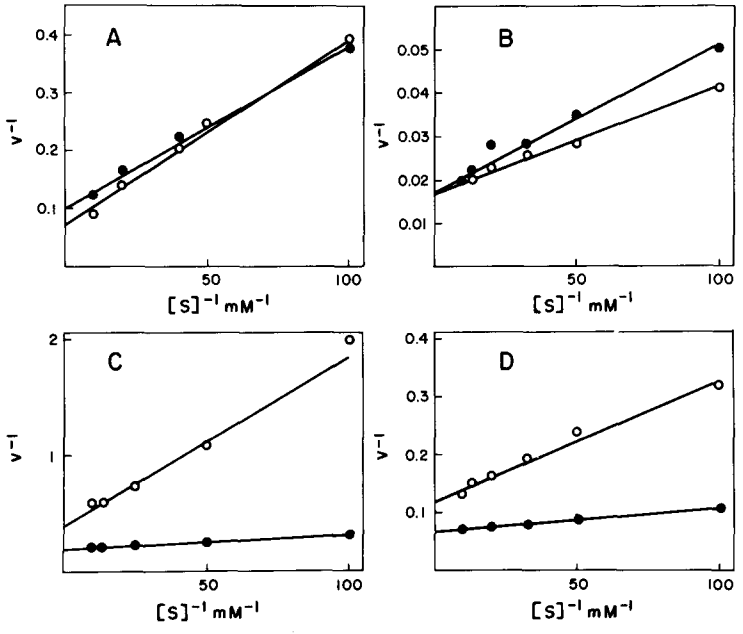

Fig. 2. Lineweaver-Burk plots for ribosylation of purines at pH 7.4 (A and B) and pH 5.0 (C and D). Sarcoma 180 cell extract and 1 mM ribose-1-phosphate were used in all K_m determinations. (A) and (C): ribosylation of Ade (•) and N^6-furfuryladenine (○) was measured at pH 7.4 (63 mM glycylglycine) and pH 5.0 (63 mM sodium citrate), respectively, using 74 μg of protein. Time of incubation was 3 and 6 min, respectively. V represents nmol of Ino + Ado or N^6-furfuryladenosine formed/mg protein/min. (B) and (D): ribosylation of Hyp (○) and Gua (•) was measured using 8 and 40 μg of protein at pH 7.4 (glycylglycine) and pH 5 (sodium citrate), respectively. Time of incubation was 3 min. For details see Materials and Methods. V represents nmol of Ino or Guo formed/mg protein/min.

170 μM for the calf spleen enzyme [25].

Nucleoside phosphorylase activity of rat liver extracts. In 63 mM glycylglycine, pH 7.4, the specific activity for ribosylation of adenine, hypoxanthine and guanine was 0.53, 239 and 190 nmol/mg protein/min, respectively. As in the case of Sarcoma 180 cells the ribosylation of all purines was dependent on the addition of ribose-1-phosphate. In the absence of ribose-1-phosphate and using 40 μg rat liver protein (2 min incubation) no significant conversion of hypoxanthine to xanthine or uric acid was detected, due to the low protein concentration used in the assay.

Nucleoside phosphorylase activity vs pH. Fig. 3A shows the effect of pH on ribosylating activity of Sarcoma 180 extract towards 6-amino and 6-oxopurines. The activity towards 6-oxopurines decreased more rapidly with decreasing pH than the activity towards 6-aminopurines. Thus, the ratio of activity for 6-oxo/6-aminopurines at pH 4.5 was about 0.5 as compared to 7 at pH 7.4. In contrast the ratio for hypoxanthine/guanine or adenine/N^6-furfuryladenine remained nearly constant.

Results of similar experiments obtained in the case of rat liver are presented as a semilogarithmic plot in Fig. 3B. In contrast to Sarcoma 180 extract the rate of ribosylation of adenine by rat liver extract was about 2-fold higher at pH 5—5.5 than at pH 7.4. As in the case of Sarcoma 180, the hypoxanthine-

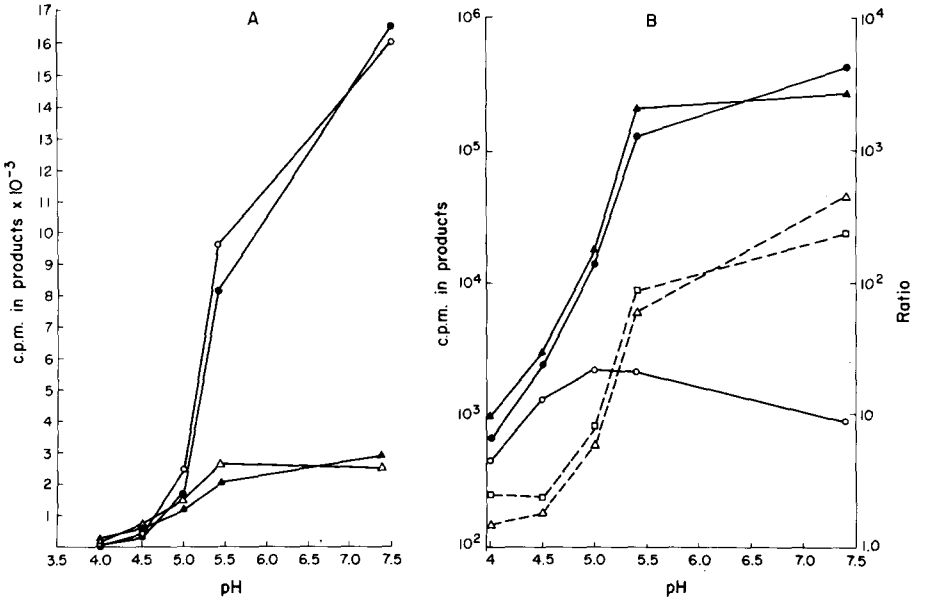

Fig. 3. (A) Effect of pH on the ribosylation of purines by Sarcoma 180 extract. Ribosylation of purines was measured at pH 7.4 (63 mM glycylglycine) and pH 4 to 5.4 (63 mM sodium citrate) using 0.2 mg protein. Incubation was for 10 min in the case of Ade and N^6-furfuryladenine and 1—10 min in the case of Hyp and Gua. The ordinate represents the cpm in products calculated for 10 min incubation. ○, Gua; ●, Hyp; △, Ade; ▲, N^6-furfuryladenine. (B) Effect of pH on ribosylation of purines by rat liver extract. Ribosylation of Hyp and Gua at pH 5.4 and 7.4 was measured using 40 μg protein (1—2 min incubation) while at other pH values and with Ade, 1 mg protein (5—10 min incubation) was used. The ordinate represents cpm in products calculated for 1 mg protein and 10 min incubation. For details see Legend to Fig. 3A and Materials and Methods ●———●, Hyp; ▲———▲, Gua; ○———○, Ade; △- - - - -△, ratio of Hyp/Ade and □- - - - -□, ratio of Gua/Ade.

guanine ribosylating activity decreased more rapidly with decreasing pH than adenine ribosylating activity. However the decrease was even more marked than in the case of Sarcoma 180. Thus the ratio of activity for 6-oxopurines/adenine at pH 4.0 was about 2 as compared to 250—460 at pH 7.4. In contrast the ratio for guanine/hypoxanthine varied only from 0.7—2.3 at these pHs. At pH 5.0 and below, because of low hypoxanthine-guanine ribosylating activity, about 1 mg liver extract protein (as compared to 40 μg at pH 5.4 and 7.4) and 5 min incubations (as compared to 1 min) were used. The possibility that xanthine oxidase present in the crude extract would deplete the substrate for ribosylation by converting hypoxanthine to xanthine and uric acid and thereby indirectly affect hypoxanthine-guanine ribosylating activity was considered. At pH 5.0, in the absence of ribose-1-phosphate but under otherwise identical conditions, less than 6% of hypoxanthine was converted to xanthine and uric acid.

Inhibition by p-chloromercuribenzoate. As observed with several purine nucleoside phosphorylase preparations [3], the enzyme activity of Sarcoma 180 extract towards 6-amino and 6-oxopurines was inhibited by 30 min initial incubation with *p*-chloromercuribenzoate. However, the activity towards 6-oxopurines as a group was more affected than the activity towards 6-amino-

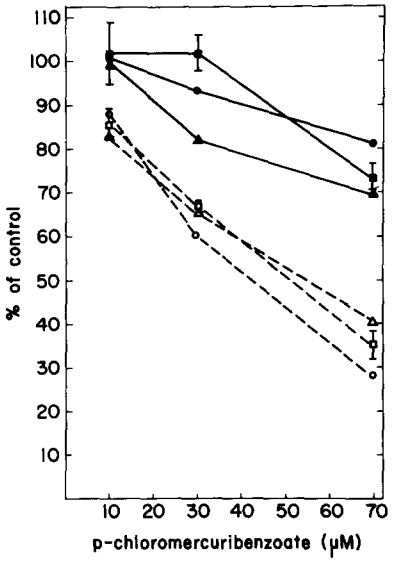

Fig. 4. Inhibition of purine nucleoside phosphorylase by *p*-chloromercuribenzoate. Sacroma 180 cell extract (~0.8 mg protein), 125 mM Tris, pH 7.4 and *p*-chloromercuribenzoate (total vol. 0.4 ml) were initially incubated at 35°C for 30 min. 20 μl aliquots of this mixture were assayed for enzyme activity with different substrates as described in Materials and Methods. Incubation was for 4 and 16 min in the case of 6-oxo and 6-amino compounds, respectively. Control consisted of Sarcoma 180 extract incubated under identical conditions in the absence of *p*-chloromercuribenzoate. Enzyme activity determined with Ade, ■———■; N^6-furfuryladenine, ●———●; N^6-(Δ^2-isopentenyl)adenosine, ▲———▲; Hyp, □- - - - -□; Guo, ○- - - - -○ and Gua △- - - - -△. Bars represent standard deviation (3 or more experiments) in the case of Ade and Hyp.

purines ($p < 0.0027$, see Fig. 4). The activity of *p*-chloromercuribenzoate free controls was not affected by 30 min initial incubation.

Heat inactivation and specificity of protection by substrates. Heating of Sarcoma 180 extract at 60°C for 20 min caused >90% loss of enzyme activity towards 6-amino and 6-oxo purines. Presence of adenine selectively protected the activity towards adenine as the substrate against heat inactivation (Table I). Thus only 28—52% activity towards adenine was lost as opposed to >90% towards hypoxanthine or guanine. Even with the heated extract the major product of adenine ribosylation was inosine because adenosine deaminase was stable to heating at 60°C. Calf serum adenosine deaminase is also known to withstand heating at 60°C for 30 min [26]. Surprisingly, heating with adenine did not protect the ribosylating activity towards N^6-furfuryladenine or phosphorolytic activity towards N^6-(Δ^2-isopentenyl)adenosine. These activities were not protected even by their own presence in the heating mixture. On heating with 0.8 mM hypoxanthine 28—32% of the ribosylating activity towards hypoxanthine and guanine was lost as opposed to >90% towards adenine, N^6-furfuryladenine and N^6-(Δ^2-isopentenyl)adenosine. The ratio of activity for hypoxanthine/adenine in the nonheated extract was 7—12. On heating with adenine it decreased to 1.1—1.6 while on heating with hypoxanthine it increased to about 100.

Similarly, on heating of rat liver extract at 60°C for 20 min, over 90% of the ribosylating activity towards adenine, hypoxanthine and guanine was lost.

TABLE I

HEAT INACTIVATION OF PURINE NUCLEOSIDE PHOSPHORYLASE

Sarcoma 180 (~4 mg protein) or rat liver (~9 mg protein) extract in 100 mM Tris, pH 7.4 was heated for 20 min at 60°C with or without the addition of compounds shown (total vol. 1 ml). After cooling in ice the solution was dialyzed against 200 vol. of 5 mM Tris buffer, pH 7.4, for 15 h changing the buffer 3 times. Enzyme activity of the dialyzed extracts (50 µl aliquot) was determined using glycylglycine, pH 7.4, and sodium-citrate, pH 5.0 buffers in the case of Sarcoma 180 and rat liver extracts, respectively. Other conditions were as described in Materials and Methods. The number of experiments is shown in the parentheses.

Cell extract		Extract heated in presence of 0.8 mM	cpm in products (% of non heated extract) Purine substrate:				
			Adenine	N^6-Furfuryladenine	N^6-(Δ^2-isopentenyl) adenosine	Hypoxanthine	Guanine
Sarcoma 180	(2)	—	<10	<10	<10	<10	<10
Rat liver	(2)	—	<10	—	—	<5	<5
Sarcoma 180	(5)	Adenine	48—72	<10	<10	<10	<10
Rat liver	(3)	Adenine	47—95	—	—	<5	<5
Sarcoma 180	(2)	N^6-Furfuryladenine	—	<10	<10	—	—
Sarcoma 180	(2)	N^6-(Δ^2-isopentenyl) adenosine	—	<10	<10	—	—
Sarcoma 180	(2)	Hypoxanthine	<10	<10	<10	70—72	70—75
Rat liver	(3)	Hypoxanthine	35—41	—	—	67—78	73—80

The presence of 0.8 mM adenine selectively protected the enzyme against heat inactivation. Thus only 5—53% ribosylating activity towards adenine was lost as opposed to more than 90% towards hypoxanthine and guanine. On the other hand heating in the presence of hypoxanthine did not as selectively protect the enzyme activity. Thus, in presence of hypoxanthine only 20—33% activity towards hypoxanthine and guanine was lost as compared to 59—65% towards adenine. Thus the ratio of activity for hypoxanthine/adenine which in the case of non-heated extract was about 60 (pH 5.4) decreased to 2—4 on heating with adenine and increased to 100—200 on heating with hypoxanthine.

Mixed substrate experiments. The initial rates of ribosylation of individual purines and a mixture of these (2 at a time) were always measured at the same time with a view to obtain comparable experimental conditions. The rate was measured at 2 time points and the average of these two varied less than ±10%. At pH 5.0 the total rate of ribosylation of hypoxanthine + guanine (125 µM each) was about equal to that of guanine alone (Fig. 5A). The concentrations of hypoxanthine and guanine used were 7 and 25 times their K_m values respectively and thus approached saturation. At pH 7.4 the rates of ribosylation of hypoxanthine, guanine or a mixture of these were about equal (Fig. 5A). It is quite clear that no additive rate effects could be observed with a mixture of hypoxanthine or guanine at either pH. In contrast, at pH 5.0 the total rate of ribosylation of a mixture of adenine + hypoxanthine (125 µM each, approaching saturation) was found to be almost equal to the sum of their individual rates as measured separately (Fig. 5C). Additive rate effects were also observed for a mixture of adenine + guanine (Fig. 5D). The rates of ribosylation of a mixture of adenine + hypoxanthine or guanine by Sarcoma 180 extract were

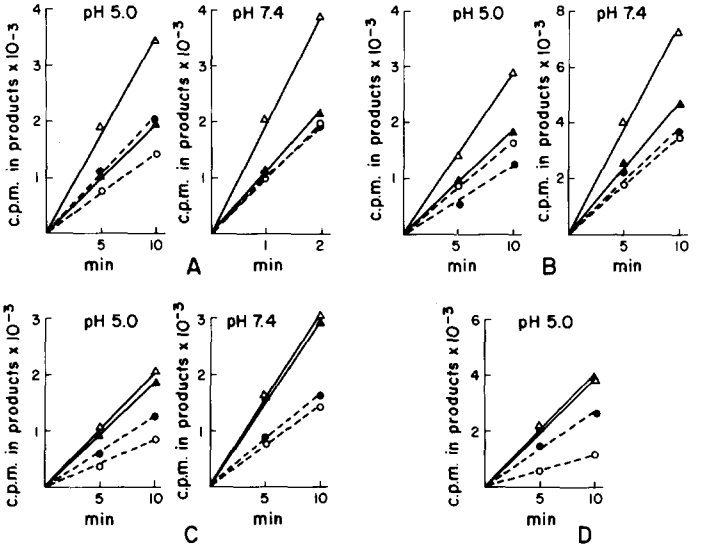

Fig. 5. Mixed substrate experiments. The rates of ribosylation of individual purines and a mixture of these by Sarcoma 180 cell extract (0.1—0.2 mg protein) were measured at pH 7.4 (63 mM glycylglycine) and at pH 5.0 (63 mM sodium citrate). The concentration of each purine used was 125 μM except for N^6-furfuryladenine, which was 250 μM. For details see Materials and Methods. (A) ○------○, Hyp; ●------●, Gua; ▲———▲, Hyp + Gua (observed) and △———△ Hyp + Gua (sum of individual rates). (B) ○------○, Ade; ●------●, N^6-furfuryladenine; ▲———▲, Ade + N^6-furfuryladenine (observed) and △———△ Ade + N^6-furfuryladenine (sum of individual rates). (C) ○------○, Ade; ●------●, Hyp; ▲———▲, Ade + Hyp (observed) and △———△, Ade + Hyp (sum of individual rates). Sarcoma 180 cell extract heat inactivated in presence of Ade was used as the source of enzyme in the experiments carried out at pH 7.4. (D) ○------○, Ade; ●------●, Gua; ▲———▲, Ade + Gua (observed) and △———△, Ade + Gua (sum of individual rates).

not measured at pH 7.4 because at this pH, the rate with adenine is only about 10% of that of hypoxanthine or guanine. Such results would appear inconclusive. This difficulty was avoided by using a cell extract heat inactivated in the presence of adenine (see Legend to Table I) as the source of the enzyme. Using this preparation also, the total rate of ribosylation of a mixture of adenine + hypoxanthine at pH 7.4 was found to be clearly additive (Fig. 5C). The total rate of ribosylation of a mixture of 125 μM adenine + 250 μM N^6-furfuryladenine (K_m, 67μM) was only slightly higher than their individual rates, but clear-cut additive effects as in the case of adenine + hypoxanthine were not observed.

Product inhibition studies. The results of the inhibition of the ribosylation of purines by the products (or alternate product) of the reaction are shown in Table II. 2-Chloroadenosine was used in these experiments instead of adenosine because it was felt that the deamination of adenosine by adenosine deaminase present in Sarcoma 180 extract would produce inosine and thereby affect the results obtained. 2-Chloroadenosine is a good inhibitor of adenosine deaminase [27] but is expected to be a very poor substrate of the enzyme [28]. It could be seen from Table II that inosine selectively inhibited the ribosylation of hypoxanthine or guanine but had no significant effect on the ribosylation of adenine and N^6-furfuryladenine except at a high concentration (2.5 mM). On

TABLE II

PRODUCT INHIBITION OF THE RIBOSYLATION OF PURINES

The ribosylation of 125 μM purine was measured in the absence and presence of inhibitors as described in Materials and Methods. With Sarcoma 180 extract, 200 μg protein and 63 mM glycylglycine buffer, pH 7.4, were used while with rat liver extract 1 mg protein and 63 mM sodium-citrate buffer, pH 5.0 were used. The data represents the average of 2 separate experiments (which did not vary more than 10%) carried out with 2 different extracts each of Sarcoma 180 and rat liver. In controls cpm in purine nucleosides formed were about 2000.

	Concentration (mM)	Cell extract	% Inhibition of the Ribosylation Purines			
			Adenine	N^6-Furfuryladenine	Hypoxanthine	Guanine
Inosine	0.63	Sarcoma 180	<5	<5	28	13
	1.25	Sarcoma 180	5	5	42	22
	2.5	Sarcoma 180	17	23	61	45
	1.25	Rat liver	11	—	38	—
	2.5	Rat liver	21	—	56	—
2-Chloroadenosine	0.63	Sarcoma 180	29	29	<5	<5
	1.25	Sarcoma 180	44	44	<5	<5
	2.5	Sarcoma 180	65	60	5	7
	5.0	Sarcoma 180	77	70	12	15
	2.5	Rat liver	32	—	<5	—
	5.0	Rat liver	44	—	<5	—

the other hand, 2-chloroadenosine selectively caused inhibition of ribosylation of adenine and N^6-furfuryladenine but had no significant effect on the ribosylation of hypoxanthine or guanine except at 5 mM. Similar results were also obtained in the case of rat liver extract (Table II). It should be noted that unlike in the other experiments, adenosine was the major product of adenine ribosylation in the presence of inosine or 2-chloroadenosine (inhibitors of adenosine deaminase). Thus in presence of 2.5 mM 2-chloroadenosine, about 80% of the radioactivity in the products was present as adenosine and only 20% as inosine. N^6-furfuryladenosine, the product of N^6-furfuryladenine ribosylation, is not a substrate of adenosine deaminase [23].

Discussion

The specific activity of Sarcoma 180 extract for the ribosylation of adenine and N^6-substituted adenines, although about 10-fold lower than that for hypoxanthine or guanine is comparable to or even higher than that of several enzymes involved in purine metabolism such as adenine and hypoxanthine phosphoribosyl transferases, adenosine kinase, IMP dehydrogenase, adenylosuccinate synthetase and phosphoribosyl pyrophosphate synthetase [21]. The ratio of adenine/hypoxanthine ribosylation for Sarcoma 180 cells is markedly different from that of rat liver and other mammalian tissues [3]. This high relative activity prompted us to test whether the phosphorolysis of adenosine and inosine or guanosine is catalyzed by a single enzyme. pH vs activity curves and *p*-chloromercuribenzoate inhibition experiments indicate that in the case of both Sarcoma 180 and rat liver, the enzyme activity responsible for the

ribosylation of 6-aminopurines (or phosphorolysis of 6-aminopurine nucleosides) is distinct from that which catalyzes the ribosylation of 6-oxopurines. The selective protection of adenine ribosylating activity against heat inactivation by adenine and of hypoxanthine-guanine ribosylating activity by hypoxanthine supports this conclusion. However the failure of adenine to protect the N^6-(Δ^2-isopentenyl)adenosine phosphorolytic or N^6-furfuryladenine ribosylating activity against heat inactivation might imply that the phosphorolysis of adenosine and N^6-substituted adenosines may be catalyzed by two separate enzyme activities. In view of this, the less than additive effects observed with a mixture of adenine + N^6-furfuryladenine (Fig. 5B) could be reconciled by the competition of adenine at N^6-furfuryladenine catalytic site and vice versa. Alternatively, the lack of protection by N^6-substituted adenines could be due to the lower affinity of these compounds for the active site.

It is of interest that N^6-substituted adenosines are ubiquitously present in mammalian cells as components of tRNA [29]. The free nucleosides which are not synthesized per se possess potent growth regulatory properties and are released into the cell during tRNA turnover. Should these get trapped inside the cells after conversion to negatively charged nucleotides by adenosine kinase, deleterious effects could result [19]. Thus the existence of an enzymic activity to catabolize these compounds to biologically inactive free bases could have physiological significance.

The additive rate effects observed with a mixture of nearly saturating concentrations of adenine + hypoxanthine or guanine but not of hypoxanthine + guanine strongly supports the view that the enzyme activity catalyzing the ribosylation of adenine is distinct from that catalyzing the ribosylation of hypoxanthine or guanine [30]. Product inhibition studies lend further support to this view. Thus, 2-chloro-adenosine selectively inhibited the ribosylation of adenine and N^6-furfuryladenine, while inosine selectively inhibited the ribosylation of hypoxanthine and guanine. In conclusion, these findings clearly indicate the presence of at least two distinct catalytic sites, if not two different proteins, for the ribosylation of 6-amino and 6-oxopurines. Purification of the enzymes(s) from Sarcoma 180 cells to resolve the question of multiple catalytic sites or enzymes is currently in progress. Adenosine phosphorylase activity of *Schistosoma mansoni* also appears to be distinct from inosine phosphorylase [31].

Zimmerman et al. [4] recently reported that extracts of several rat tissues catalyze a very slow ribosylation of adenine. According to our estimations, adenine and hypoxanthine (125 μM each) ribosylating activity of rat liver extract is 0.5 and 239 nmol/mg protein/min, respectively, at pH 7.4. Our values are 5—6 times higher than Zimmerman's values, at least partly because our assay temperature was 35°C as opposed to 30°C. However, the ratio of 250—460 found for hypoxanthine/adenine ribosylation was comparable to the ratio of 274 observed by Zimmerman et al. [4]. On the basis of the low activity with adenine as compared to hypoxanthine, Zimmerman et al. [4] and Parks and Agarwal [3] concluded that it is unlikely that purine nucleoside phosphorylase plays a significant role in the metabolism of adenosine in mammalian cells. Assuming that adenosine phosphorylase is distinct from inosine-guanosine phosphorylase and the activity observed in cell extracts (0.5 nmol/

mg protein/min at pH 7.4) were fully expressed in rat liver, in vivo, one can calculate that for a 200 gm rat with a 10 gm liver, the potential for adenosine phosphorolysis could be as high as 300 mg/kg body wt/day. On a purine-free diet, the average uric acid output of an adult human is only about 6 mg/kg body wt/day. Even hyperuricemic children suffering from Lesch Nyhan syndrome (who lack hypoxanthine-guanine phosphoribosyl transferase), excrete about 47 mg uric acid/kg body wt/day [2]. Based on this figure, Murray [2] estimated that about 40 mg/kg body wt of hypoxanthine or guanine is normally recovered. The high potential capacity for adenosine phosphorylysis suggests a possibility that substantial amounts of adenine are normally produced in various tissues which may be recovered by adenine phosphoribosyl transferase.

It is of interest to note that reutilization of adenosine via adenosine kinase or phosphorylase (see Fig. 1) should be more economical than adenosine deaminase, since conversion of inosine back to AMP would require one ATP and GTP molecule as opposed to only one ATP molecule in the former two reactions (assuming ribose-1-phosphate generated during nucleoside phosphorolysis is utilized for synthesizing phosphoribosyl pyrophosphate). In view of this, adenosine phosphorylase may have special significance in rapidly dividing tissues like bone marrow. Purine conservation by recovery could be an important process in the economy of this tissue as it lacks the capacity to synthesize purines de novo [7]. The enzyme activity may also play a role in tissues low in adenosine deaminase activity [32] or in catabolizing cytotoxic adenosine analogs like N^6-(Δ^2-isopentenyl)adenosine which are released into the cell due to tRNA turnover. In summary, to the best of my knowledge, these are the first findings in any mammalian cells of a purine nucleoside phosphorylase activity which appears to be specific for adenosine and distinct from inosine-guanosine phosphorylase activity.

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