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5′-METHYLTHIOADENOSINE PHOSPHORYLASE—I

SUBSTRATE ACTIVITY OF 5′-DEOXYADENOSINE WITH THE ENZYME FROM SARCOMA 180 CELLS*

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Abstract—5′-Deoxy-5′-methylthioadenosine phosphorylase (MTA phosphorylase), an enzyme involved in the salvage of adenine moieties from 5′-deoxy-5′-methylthioadenosine (MTA) produced primarily during polyamine biosynthesis, is present in Sarcoma 180 cells (0.0026 ± 0.0002 μM units/mg cytosol protein). 5′-Deoxyadenosine (5′-dAdo), an adenosine analog previously thought not to be metabolizable, has been shown [D. Hunting and J. F. Henderson, *Biochem. Pharmac.* **27**, 2163 (1978)] to have a number of biochemical effects on Ehrlich ascites cells. We have now found that 5′-dAdo is a substrate for the MTA phosphorylase from Sarcoma 180 cells, yielding free adenine and 5-deoxyribose-1-phosphate. The reaction was reversible and totally dependent upon phosphate. Evidence that MTA phosphorylase is responsible for 5′-dAdo phosphorylase activity includes the following: (1) Sarcoma 180 MTA phosphorylase preparations did not show additive rates of adenine production in the presence of saturating concentrations of both 5′-dAdo and MTA; (2) double-reciprocal plots of the rates of adenine formation from 5′-dAdo by Sarcoma 180 enzyme preparations in the presence of MTA displayed a pattern characteristic of alternative, competing substrates; (3) the rate of depletion of 5′-dAdo by Sarcoma 180 preparations was inhibited by the presence of MTA; (4) the K_i value of a competitive inhibitor of Sarcoma 180 MTA phosphorylase, 5′-deoxy-5′-chloroformycin, was the same when either MTA or 5′-dAdo was employed as substrate; and (5) the apparent K_m values of phosphate for both MTA and 5′-dAdo phosphorylase activities were identical (3.5 mM). The K_m of Sarcoma 180 MTA phosphorylase for MTA is 4 μM; the K_m for 5′-dAdo is 23 μM (V_{max} relative to MTA = 180 per cent). Incubation of Sarcoma 180 cells with either 5′-dAdo or MTA caused profound elevations of adenine nucleotides, as well as an inhibition of 5-phosphoribosyl-l-pyrophosphate (PRPP) accumulation. The reaction of 5′-dAdo with MTA phosphorylase to yield free adenine, which is then salvaged to adenine nucleotides, can account for many of the previously reported biochemical effects of 5′-dAdo, such as inhibitions of PRPP accumulation, purine *de novo* synthesis, and glycolysis that have previously been attributed to the unmetabolized nucleoside. The other product of this reaction, 5-deoxyribose-l-phosphate, may also contribute to these effects.

5′-Deoxyadenosine (5′-dAdo)‡ is an analog of adenosine that cannot be directly converted to 5′-nucleotides and has only weak substrate activity with adenosine deaminase (K_m, approximately 330 μM; V_{max} relative to adenosine, < 1 per cent [1]). Nevertheless, Hunting and Henderson [2] reported that 5′-dAdo caused several profound metabolic disturbances when incubated with Ehrlich ascites cells, including inhibitions of PRPP accumulation and purine synthesis *de novo*, and of glycolysis, with a "cross-over" at the phosphofructokinase step. It was proposed that this compound produces these metabolic perturbations at the nucleoside level. These investigators, however, failed to demonstrate direct effects of 5′-dAdo on either PRPP synthetase or phosphofructokinase.

In an attempt to explain the surprising biological activity of 5′-dAdo, our attention was drawn to an earlier report by Pegg and Williams-Ashman [3] of an enzyme in rat ventral prostate that catalyzes the phosphate-dependent cleavage of adenine from 5′-deoxy-5′-methylthioadenosine (MTA). This enzyme, now referred to as 5′-methylthioadenosine phosphorylase (MTA phosphorylase), is believed to function in polyamine biosynthesis. MTA is produced when *S*-adenosylmethionine (SAM) is decarboxylated and a propylamine group is transferred to putrescine forming spermidine, in a sequence of reactions catalyzed by SAM decarboxylase and spermidine synthase [4]. The overall reaction is:

$$\text{Putrescine} + \text{SAM} \rightarrow \text{CO}_2 + \text{spermidine} + \text{MTA} \quad (1)$$

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‡ Abbreviations used: 5′-dAdo, 5′-deoxyadenosine; ADA, adenosine deaminase (EC 3.5.4.4); APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); dCF, 2′-deoxycoformycin (*R*-3-(2-deoxy-β-D-erythropento-furanosyl)-3,6,7,8-tetrahydroimidazo [4,5-*d*] (1,3) diazepin-8-ol); MTA, 5′-deoxy-5′-methylthioadenosine; PNP, purine nucleoside phosphorylase (EC 2.4.2.1); PRPP, 5-phosphoribosyl-l-pyrophosphate; SAM, *S*-adenosyl-L-methionine; and HEPES, 4-(2-hydroxyethyl)-l-piperazine-ethanesulphonic acid.

A second mole of MTA is produced in a similar manner during spermine synthesis, in reactions catalyzed by SAM decarboxylase and spermine synthase [5]. Here the overall reaction is:

$$\text{Spermidine} + \text{SAM} \rightarrow CO_2 + \text{spermine} + \text{MTA} \quad (2)$$

MTA phosphorylase cleaves the glycosidic bond of MTA, yielding free adenine and 5-methylthioribose-1-phosphate [6], as shown in Fig. 1.

The adenine produced can then react with PRPP catalyzed by APRT, forming AMP:

$$\text{Adenine} + \text{PRPP} \rightarrow \text{AMP} + PP_i \quad (3)$$

The AMP may then be converted to polyphosphate adenine nucleotides. Thus, it is likely that MTA phosphorylase plays a crucial role in the salvage of adenine moieties from SAM (originally formed by the condensation of ATP and methionine) consumed during polyamine biosynthesis [7]. Recently, this enzyme has been found in human prostate [8], human placenta [9], a variety of rat tissues [6, 10], in several murine tumors of hematopoietic origin [11], and also in *Drosophila melanogaster* [12]; although the corresponding enzyme in most microorganisms appears to be a hydrolase [13, 14], an MTA phosphorylase has been reported in *Caldariella acidophila* [15]. The presence of MTA phosphorylase in many mammalian tissues offers a satisfactory answer to questions that have puzzled biochemists for many years, i.e. what is the source of adenine, and what is the reason for the virtually ubiquitous distribution of APRT in mammalian tissues?

The present report provides evidence that 5′-dAdo is, in fact, metabolized and that the enzyme responsible is MTA phosphorylase. This metabolism explains many of the biochemical effects of 5′-dAdo previously ascribed to the nucleoside itself [2]. Furthermore, the results of these initial studies encourage us to propose MTA phosphorylase as a chemotherapeutic target, as will be discussed in detail in other papers of this series. Preliminary reports of portions of these findings have been published [16–18].

MATERIALS AND METHODS

Materials

The following compounds were obtained from commercial sources: adenine, 2,8-dihydroxyadenine, MTA, phosphocreatine, and PRPP (Sigma Chemical Co., St. Louis, MO); AMP, ADP, and ATP (P-L Biochemicals, Milwaukee, WI). A sample of 5′-deoxyadenosine was provided by Dr. J. G. Cory of the University of South Florida. Samples of 5′-deoxyadenosine and 5′-deoxy-5′-chloroformycin were synthesized by Dr. S. H. Chu, Brown University. Dr. J. D. Stoeckler, Brown University, provided 5-deoxyribose-l-phosphate. Samples of dCF were provided by Dr. H. W. Dion of Parke-Davis, Detroit, MI, and Dr. John Douros of the National Cancer Institute. Buttermilk xanthine oxidase (Grade III), calf intestinal adenosine deaminase (ADA) Type I, rabbit muscle myokinase (adenylate kinase), and rabbit muscle creatine phosphokinase were obtained from the Sigma Chemical Co.; calf spleen purine nucleoside phosphorylase (PNP) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); human erythrocytic PNP was provided by Dr. J. D. Stoeckler; and orotidine-5′-phosphate pyrophosphorylase was purchased from P-L Biochemicals.

Spectrophotometric assay of MTA phosphorylase

MTA phosphorylase was measured by a modification of a method previously described by Pegg and Williams-Ashman [3]. The liberation of free adenine from MTA is followed by coupling the reaction to xanthine oxidase, which converts free adenine to 2,8-dihydroxyadenine, resulting in an increase in absorbance at 305 nm ($\Delta E = 15{,}500$ molar absorbancy units) [19]. Assays were performed on a Gilford recording spectrophotometer at 37°. Each cuvette contained 40 mM potassium phosphate (pH 7.4), 0.8 units of xanthine oxidase, crude or partially purified supernatant fractions of Sarcoma 180 extracts (450–675 μg protein), and 0.5 mM MTA in a 1 ml volume. The reaction mixture, excluding the enzyme preparation, was preincubated at 37° to remove any trace contaminant of adenine. Under these conditions the rate of adenine release was linear with protein concentration up to 675 μg protein/ml of reaction mixture. This assay was adequate for kinetic studies utilizing both MTA and 5′-dAdo as substrates. Enzyme preparations were preincubated with the ADA inhibitor dCF (5 μg/ml) for 20 min at 37° prior to assay to avoid an artifactual absorbancy increase in this coupled assay due to the sequential reactions of ADA, PNP, and xanthine oxidase with 5′-dAdo. At this concentration, dCF had no effect on MTA phosphorylase activity. A unit of MTA phosphorylase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of adenine per minute under the conditions of the standard assay described above. Specific activity is expressed as units of enzyme activity per milligram of protein.

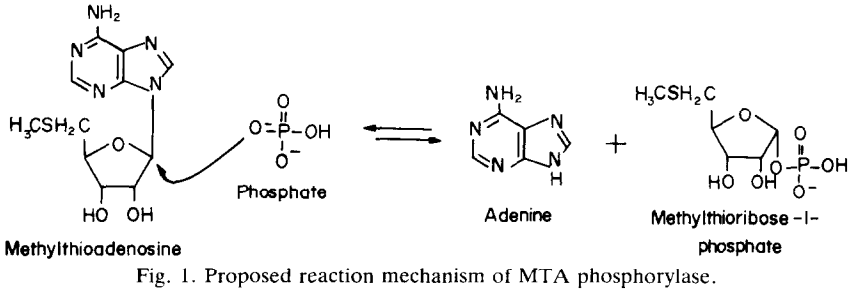

Fig. 1. Proposed reaction mechanism of MTA phosphorylase.

Reversed-phase high performance liquid chromatographic assay of MTA phosphorylase

MTA phosphorylase activity was also monitored directly by following the formation of adenine by reversed-phase high performance liquid chromatography (h.p.l.c.). MTA (0.5 mM) was incubated at 37° in a shaking water bath in the presence of 40 mM potassium phosphate (pH 7.4), and crude or partially purified Sarcoma 180 MTA phosphorylase preparations (400–600 μg protein/ml of reaction mixture). At appropriate times, aliquots were inactivated with PCA (final concentration, 4%) at 4°. After centrifugation, the supernatant fluids were neutralized to pH 6.5 to 7.5 with 5 N KOH. Following centrifugation to remove perchlorate salts, the samples were frozen (−20°) prior to reversed-phase h.p.l.c. analysis. For determination of adenine, MTA, and 5'-dAdo, the samples were chromatographed on a Varian 4200 high performance liquid chromatograph equipped with a Waters μBondapak C_{18} analytical column (4.5 mm × 30 cm). Nucleosides and bases were separated with a programmed gradient employing 10 mM potassium phosphate (pH 5.5) as the low concentrate (starting) eluent and 10 mM potassium phosphate (pH 5.5), containing 20% methanol, as the high concentrate eluent. The gradient profile consisted of a linear increase of high concentrate from 0 to 100% at a rate of 9%/min. After this, a 10-min isocratic elution at 100% high concentrate was performed, followed by a linear decrease in the level of high concentrate (−9%/min) until 0% was reached. The flow rate was 1.0 ml/min. Under these conditions, adenine can be readily separated from both 5'-dAdo and MTA, with retention times of approximately 11.5, 16.5 and 26.0 min respectively. Each of these compounds was identified and quantitated using authentic standards. To facilitate identification, the U.V. spectra of unknown peaks collected from the h.p.l.c. column were determined with a Perkin Elmer 402 spectrophotometer and compared with spectra of authentic standards. The formation of adenine as measured by this assay was linear with time (up to 15 min) and protein (up to at least 1000 μg/ml).

Anion-exchange high performance liquid chromatographic assay of MTA phosphorylase

MTA phosphorylase activity was also measured by anion-exchange h.p.l.c. Here, the adenine liberated from MTA was converted by the APRT activity present in the Sarcoma 180 enzyme preparations to AMP (see Eqn. 3), which is readily detected by anion-exchange h.p.l.c. The reaction mixture included 0.5 mM MTA, 2 mM $MgCl_2$, 50 mM potassium phosphate (pH 7.4), 1 mM PRPP, and Sarcoma 180 MTA phosphorylase preparations (300–600 μg protein) in a volume of 1 ml. The mixture was incubated at 37° in a shaking water bath, and at appropriate times aliquots were taken and prepared for anion-exchange h.p.l.c. in the same manner as described above for reversed-phase h.p.l.c. The details of the anion-exchange chromatography method used to detect AMP and other adenine nucleotides have been described [20]. Authentic AMP was used as a standard to identify and quantitate the AMP peak produced in this assay. The identity of this peak as AMP was further confirmed using a previously described peak shift method [21], in which the AMP peak was abolished by conversion to ADP and ATP in the presence of adenylate kinase, creatine phosphokinase, phosphocreatine, and ATP. This peak shift method was specific for AMP; the retention time of IMP was unaltered by treatment with these reagents. For a true determination of MTA phosphorylase activity by this method, it is essential that the indicator enzyme, APRT, not be rate-limiting. APRT activity, as measured by procedures described previously [22], was 2.6-fold higher than MTA phosphorylase activity in crude or partially purified Sarcoma 180 enzyme preparations. The formation of AMP from MTA measured by this method was linear with time for at least 60 min, and linear with protein concentration, up to 1000 μg protein/ml of reaction mixture.

Enzyme preparations

Sarcoma 180 ascites cells were obtained from female CDl mice (usually five) 6–7 days after i.p. inoculation with approximately 2.5×10^6 cells. All of the following procedures were performed at 4°. After the cells (4–8 ml packed cells) were washed several times in normal saline and freed from any erythrocytic contamination by centrifugation, they were suspended in 2 vol. of a buffer containing 10 mM HEPES (pH 7.2), 100 mM KCl, and 1 mM dithiothreitol (DTT) (HEPES–KCl–DTT buffer) and homogenized in a Potter-Elvehjem manual ground-glass homogenizer (16 strokes). The homogenate was then centrifuged at 105,000 *g* for 1 hr in a Beckman L2-65 ultracentrifuge and the pellet was discarded. The supernatant fluid was fractionated by the slow addition of solid ammonium sulfate (0.23 g/ml extract) to achieve 40% saturation. The extract was stirred for 30 min and centrifuged for 20 min at 4000 *g*. The pellet was discarded and solid ammonium sulfate (0.156 g/ml extract) was added slowly to bring the supernatant fluid to 65% saturation. Following 30 min of stirring, the solution was centrifuged again at 4000 *g* and the supernatant fraction was discarded. The remaining precipitate was dissolved in HEPES–KCl–DTT buffer (usually 3–5 ml) and the protein concentration was adjusted to 7–12 mg/ml. The solution was dialyzed against several changes of this buffer for 4 hr at 4° and stored at −20°. The results of a typical purification procedure are summarized in Table 1. The activities presented in Table 1 are approximately double those previously reported [17] for this tissue, probably due to the presence of DTT throughout purification. DTT has been shown to be required for maximal MTA phosphorylase activity in other systems [8]. Most of the studies described here employed MTA phosphorylase at the purification level of Fraction II (see Table 1).

Sephadex chromatography

A sample of Fraction II (0.077 units in 4.0 ml) was added onto a Sephadex G-100 column (3 cm × 66 cm) previously equilibrated with a potassium phosphate buffer (55 mM, pH 7.5) containing 1 mM DTT. Elution was performed with the same buffer at a flow rate of 0.4 ml/min, and 2-ml fractions were

Table 1. Typical partial purification of Sarcoma 180 MTA phosphorylase*

Procedure	Volume (ml)	Protein (mg/ml)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Fold purification
105,000 *g* Supernatant fluid (Fraction I)	9.2	5.8	0.15	0.028	100	1
40–65% Ammonium sulfate fraction (Fraction II)	3.3	7.3	0.14	0.057	93	2

* Experimental details are given in Materials and Methods. MTA phosphorylase activity was assayed by the xanthine oxidase-coupled spectrophotometric method (see Materials and Methods). Protein was measured as described by Bradford [23].

collected. Both MTA and 5'-dAdo phosphorylase activities were assayed spectrophotometrically; PNP activity was measured by the method of Kim *et al.* [24].

Metabolite determinations

Determination of adenine nucleotides was performed by anion-exchange h.p.l.c. as described previously [20]. Intracellular PRPP levels were measured using the method of Reem [25]. Protein concentration, unless otherwise noted, was assayed by the method of Lowry *et al.* [26], using crystalline bovine serum albumin (Pentex, Kankakee, IL) as a standard.

RESULTS

Identity of MTA and 5'-dAdo phosphorylase activities in Sarcoma 180 extracts

Demonstration of MTA phosphorylase activity in Sarcoma 180 extracts. The cleavage of free adenine from MTA as catalyzed by a Sarcoma 180 extract is shown in Fig. 2. That the reaction product was, in fact, adenine is demonstrated by a number of findings: (1) the new h.p.l.c. peak formed when Fraction II was incubated with MTA had a retention time identical to that of authentic adenine; (2) the u.v. spectrum of this peak (collected from the reversed-phase column) corresponded to that of authentic adenine ($\lambda_{max} = 260$ nm); (3) when the presumptive adenine peak was collected and treated with xanthine oxidase, the product formed had a u.v. spectrum identical to that of 2,8-dihydroxyadenine ($\lambda_{max} = 305$ nm), the known product of the reaction of this enzyme with adenine [19]; and (4) when an aliquot of Fraction I was dialyzed and treated with MTA and PRPP, the formation of AMP was readily detected by anion-exchange h.p.l.c., with the identity of AMP confirmed by an enzymic peak-shift technique specific for this nucleotide.

If an MTA phosphorylase similar to that previously described in other mammalian tissues was responsible for the observed production of adenine from MTA in Sarcoma 180 extracts, this activity should show absolute dependence on orthophosphate. No activity was observed when dialyzed aliquots of Fraction II were assayed in a phosphate-free buffer. Kinetic analysis with orthophosphate as the variable substrate yielded linear double-reciprocal plots, with an apparent K_m for orthophosphate

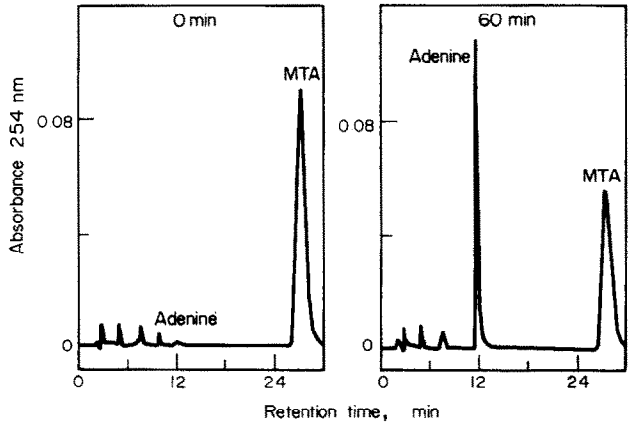

Fig. 2. Reversed-phase h.p.l.c. profiles demonstrating the liberation of adenine from MTA by partially purified Sarcoma 180 extracts (Fraction II). Fraction II (5.0 mg protein) was incubated with MTA (375 μM) and a potassium phosphate buffer (15 mM, pH 7.4) in a 2-ml volume in a shaking water bath (37°). Aliquots were prepared for reversed-phase h.p.l.c. by the procedure outlined in Material and Methods. Reversed-phase h.p.l.c. was performed on the samples as described in Materials and Methods; see text for the techniques used to identify the adenine peak.

of 3.5 mM. Sodium arsenate can replace phosphate as a substrate, as it does with other mammalian phosphorylases such as PNP [27], though the maximal activity seen at saturating arsenate concentrations is less than half that observed at saturating phosphate concentrations.

That PNP was not responsible for the phosphorolysis of MTA is based on a number of lines of evidence. MTA does not react with PNP from either calf spleen or human erythrocytes [3], as was confirmed in the present studies. Furthermore, as can be seen in Fig. 3, MTA phosphorylase activity could be cleanly separated from PNP by Sephadex G-100 chromatography.

The K_m of MTA with Fraction II was approximately 4 μM as determined by double-reciprocal plots that are linear over a concentration range of 3.0 to 100 μM. The activity of MTA phosphorylase in Sarcoma 180 cell extracts (Fraction I) was determined by the spectrophotometric assay to be 0.0026 ± 0.0002 units/mg protein, or about 0.05 units/ml of cells.

Liberation of free adenine from 5'-deoxyadenosine by Sarcoma 180 extracts. In addition to producing free adenine from MTA, Sarcoma 180 extracts were also capable of liberating adenine from 5'-dAdo in a time-dependent manner, as monitored by reversed-phase h.p.l.c. The h.p.l.c. peak produced from 5'-dAdo was identified as adenine by the same criteria described above for MTA. No adenine was formed when 5'-dAdo was incubated in the absence of cell extracts. 5'-dAdo is not a substrate for PNP from either calf spleen or human erythrocytes, indicating that this enzyme probably was not responsible for the observed liberation of adenine from 5'-dAdo. Furthermore, 5'-dAdo cleavage activity was readily separated from PNP activity by Sephadex G-100 chromatography (Fig. 3).

Two lines of evidence indicate that 5'-dAdo was cleaved by a phosphorolytic mechanism. First, the formation of adenine from 5'-dAdo, as measured by the spectrophotometric assay was absolutely dependent on orthophosphate: the apparent K_m of phosphate in this reaction was about 3.5 mM, i.e. the same value as that determined with MTA. Second, incubation of aliquots of Fraction II with 0.4 mM 5-deoxyribose-1-phosphate (prepared as described elsewhere [1]) and an excess of adenine in the absence of inorganic phosphate resulted in the formation of a compound that on reversed-phase h.p.l.c. had a retention time identical to that of authentic 5'-dAdo. The reversibility of 5'-dAdo cleavage under these conditions argues strongly for a phosphorolytic mechanism: a hydrolase would not be reversible.

The apparent K_m of 5'-dAdo with Fraction II was approximately 23 μM, and the V_{max} of 5'-dAdo relative to MTA was about 180 per cent. Kinetic parameters were measured by the xanthine oxidase-coupled spectrophotometric procedure.

Evidence that MTA and 5'-dAdo are alternative substrates for MTA phosphorylase. The ability of Sarcoma 180 cell extracts to liberate adenine from both 5'-dAdo and MTA suggested that 5'-dAdo is an alternative substrate for MTA phosphorylase. A number of lines of evidence support this hypothesis.

First, when aliquots of Fraction II were incubated simultaneously with saturating concentrations of both MTA (200 μM) and 5'-dAdo (300 μM), the rate of adenine formation (nmoles/min/mg protein) (3.0 ± 0.1) was intermediate between the rates obtained in the presence of saturating concentrations of either MTA alone (2.4 ± 0.1) or 5'-dAdo alone (4.4 ± 0.3). If MTA and 5'-dAdo had been metabolized by two separate enzymes, the rate of adenine formation at saturating concentrations of both substrates would have been additive.

More rigorous evidence indicating that MTA and 5'-dAdo are, in fact, alternative, competing substrates is depicted in Fig. 4. In this double-reciprocal plot, 5'-dAdo is the variable substrate, whose rate of cleavage to adenine was determined in the absence and in the presence of two fixed concentrations of the competing substrate, MTA. The pattern observed, as discussed by Cha [28], is exemplary of two competing substrates that have significantly different V_{max} values (the V_{max} of 5'-dAdo is 1.8-fold higher than that of MTA). Simply stated, at high

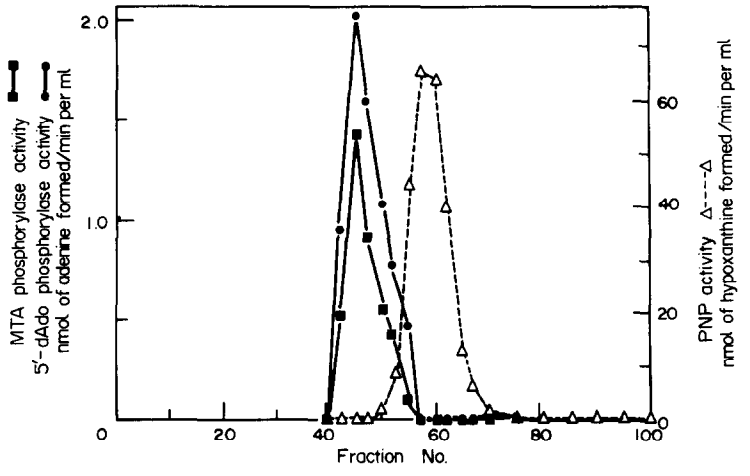

Fig. 3. Separation of purine nucleoside phosphorylase activity from MTA and 5'-dAdo phosphorylase activities by Sephadex G-100 chromatography. Experimental details are described in Materials and Methods.

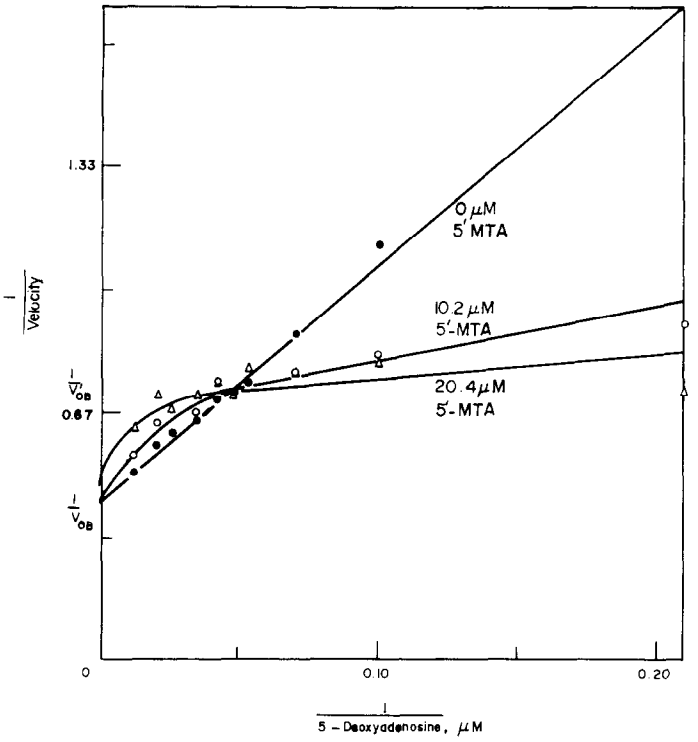

Fig. 4. Double-reciprocal plot demonstrating the alternative, competing substrate pattern observed with MTA and 5'-dAdo. Reaction mixtures (1 ml) for the xanthine oxidase-coupled spectrophotometric assay contained dCF-treated (5 μg/ml) Fraction II (290 μg protein), 20 mM potassium phosphate (pH 7.4), and 0.8 units xanthine oxidase; 5'-dAdo and MTA were added at the concentrations indicated. The *y*-axis, l/velocity, is defined as 1/nmoles of adenine formed/min/mg protein; $1/V'_{ob}$ represents the reciprocal of the V_{max} of MTA phosphorylase for MTA as obtained graphically from the intersection point extrapolated to the *y*-axis; $1/V_{ob}$ represents the reciprocal of the V_{max} of MTA phosphorylase for 5'-dAdo as obtained graphically from the *y*-intercept of the line obtained in the absence of MTA.

concentrations of the higher V_{max} substrate (5'-dAdo), the presence of the lower V_{max} substrate (MTA) will produce an apparent inhibition when it (MTA) binds to the enzyme and reacts at a relatively slower rate; at low concentrations of the higher V_{max} substrate, the presence of the lower V_{max} substrate will produce an apparent stimulation of the velocity, due to the increased availability of total substrate. The *x*-coordinate of the point of intersection, i.e. where the presence of the lower V_{max} substrate causes neither apparent inhibition nor stimulation, may be estimated from the following equation [28, 29]:

$$\frac{1}{[S]} = \frac{1}{K}\left[\frac{V - V'}{V'}\right]$$

where S is the higher V_{max} substrate (5'-dAdo), V and V' are the apparent V_{max} values for the higher and lower V_{max} substrates respectively (in this case, 5'-dAdo and MTA), and K is the Michaelis constant of the higher V_{max} substrate (5'-dAdo). Substituting the appropriate kinetic values for MTA and 5'-dAdo into this equation, the *x*-coordinate of the predicted point of intersection (in terms of 1/[5'-dAdo]) is 0.036, close to the observed value (see Fig. 4). The *y*-coordinate of this intersection point represents $1/V'$ [28]: in this case, the reciprocal of the V_{max} for MTA. As can be seen in Fig. 4, the observed intersection point gives a $1/V'$ value of 0.74, which is 1.72-fold higher than the observed $1/V$ value of 5'-dAdo, 0.43. This ratio is close to the expected $1/V'$:$1/V$ value (1.80) based on the known relative V_{max} values of the two substrates. The above kinetic data support the hypothesis that MTA and 5'-dAdo are alternative substrates for a single enzyme, with each substrate competing for the same catalytic site(s).

If MTA and 5'-dAdo are both substrates of MTA phosphorylase, the presence of MTA should inhibit the reaction of this enzyme with 5'-dAdo. Through the use of reversed-phase h.p.l.c., the rate of 5'-dAdo disappearance mediated by Fraction II could be followed both in the presence and absence of MTA. When incubated alone at 25 μM (approximately the K_m value of 5'-dAdo with MTA phosphorylase), 54 per cent of the original 5'-dAdo remained after 30 min. In the presence of 25 μM MTA (about six times its K_m value), however, the rate of depletion of 5'-dAdo (25 μM) was substantially reduced, with 80 per cent of the original 5'-dAdo remaining after 30 min. In contrast, the presence of 5'-dAdo (25 μM) in the reaction mixture had little effect on the rate of depletion of 25 μM MTA. These results may be explained in terms of two substrates with different K_m values competing for the same enzyme. When both substrates were present, the substrate with the lower K_m value, MTA, was

preferentially phosphorolyzed, sparing the substrate with the higher K_m value, 5'-dAdo. Thus, this evidence corroborates the conclusions of the alternative substrate kinetic studies (see above) in which the rates of product formation were followed rather than the rates of substrate depletion, as were monitored in these experiments.

If 5'-dAdo and MTA are both substrates for the same enzyme, then a competitive inhibitor of the enzyme should have the same K_i value when tested with either substrate. As will be documented in detail elsewhere, double-reciprocal plots show that 5'-deoxy-5'-chloroformycin is a competitive inhibitor of MTA phosphorylase from Sarcoma 180 cells. As can be seen in Fig. 5, identical K_i values of 5'-deoxy-5'-chloroformycin with MTA phosphorylase (0.3 μM) were obtained when either MTA or 5'-dAdo served as the substrate. Since it is unlikely than an inhibitor would have the same K_i value for two distinct enzymes, these results further support the postulate that MTA and 5'-dAdo phosphorylases are one enzyme.

In agreement with this concept are the identical K_m values of the Sarcoma 180 enzyme for phosphate (3.5 mM) when determined with either 5'-dAdo or MTA as the fixed substrate. Further, although MTA phosphorylase has not been extensively purified from Sarcoma 180 cells, the purification steps used to date (ammonium sulfate fractionation and Sephadex G-100 chromatography) have failed to demonstrate separate phosphorylase activities for 5'-dAdo and MTA (see Fig. 3).

Despite the use of relatively impure enzyme preparations in these studies, all evidence indicates that they are suitable for the kinetic studies described above. There is no evidence for any side reactions that would affect either substrate or product concentration. Under the conditions employed, rates were linear with regard to both time and protein concentration, and all double reciprocal and Dixon plots used to calculate kinetic parameters were linear. Obviously, further purification of MTA phosphorylase is desirable, to further characterize this enzyme.

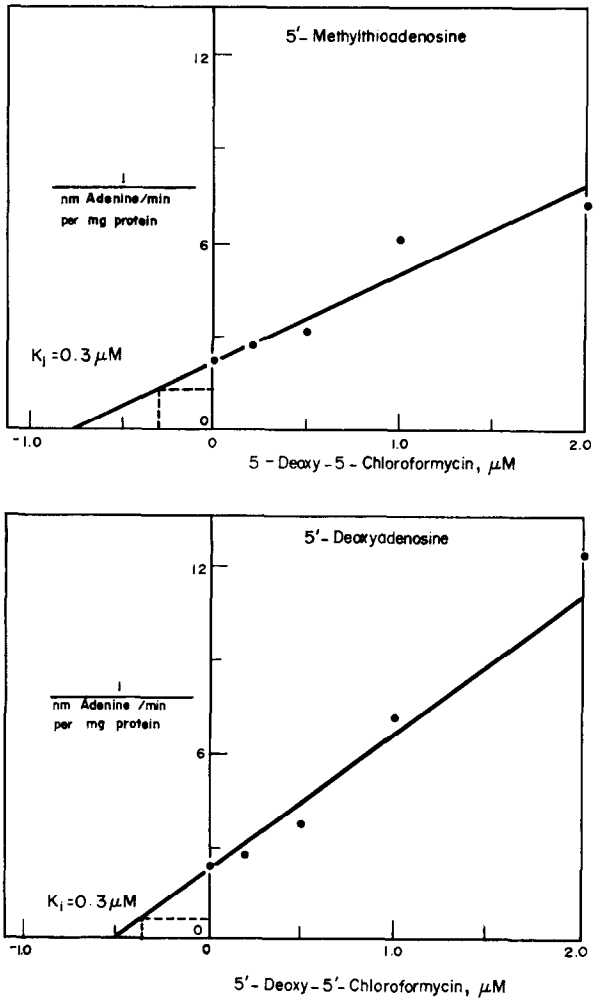

Fig. 5. Dixon plots for the inhibition of Sarcoma 180 MTA phosphorylase by 5'-deoxy-5'-chloroformycin. Upper panel: MTA as substrate. Lower panel: 5'-dAdo as substrate. Reaction mixtures (1 ml), assayed by the xanthine oxidase-coupled spectrophotometric method, contained dCF-treated (5 μg/ml) Fraction II (700 μg protein), 15 mM potassium phosphate (pH 7.4), 0.8 units xanthine oxidase, either 8.5 μM MTA or 15 μM 5'-dAdo, and various amounts of 5'-deoxy-5'-chloroformycin as indicated. Data were plotted according to the method of Dixon and Webb [30].

Metabolic effects of 5'-dAdo and MTA on intact Sarcoma 180 cells

Effects on adenine nucleotide pools. Incubation of Sarcoma 180 cells *in vitro* with MTA or 5'-dAdo led to a rapid increase of intracellular adenine nucleotides, especially ATP (Fig. 6). When Sarcoma 180 cells were incubated for 3 hr with 5'-dAdo (following pretreatment with dCF to eliminate the possible deamination of 5'-dAdo by ADA), the concentrations of ATP, ADP, and AMP increased 2.5-, 2.4- and 4.6-fold, respectively, above the corresponding nucleotide concentrations of control cells (pretreated with dCF alone) (Fig. 6, upper panel). In cells incubated in the same manner with MTA for 3 hr (Fig. 6, lower panel), the concentrations of ATP, ADP, and AMP increased 2.5, 2.8, and 5.3 times, respectively, over those of control cells.

The initial average rate of total adenine nucleotide accumulation (AMP + ADP + ATP) during the first 30 min was over two times faster in the 5'-dAdo-treated cells (0.143 μmole/min/ml of cells) than in the MTA-treated cells (0.069 μmole/min/ml of cells). Nucleotide incorporation from 5'-dAdo occurred rapidly, with 80 per cent of the total eventual nucleotide formation occurring by 1 hr. Nucleotide accumulation from MTA was more gradual, with only 45 per cent of the total eventual nucleotide formation occurring by 1 hr. In both cases, however, nucleotide formation did not continue after 2 hr; i.e. after approximately 50 per cent of the original amounts of both 5'-dAdo and MTA were incorporated into nucleotides.

Effects on PRPP accumulation. In agreement with earlier studies with Ehrlich ascites cells [31, 32],

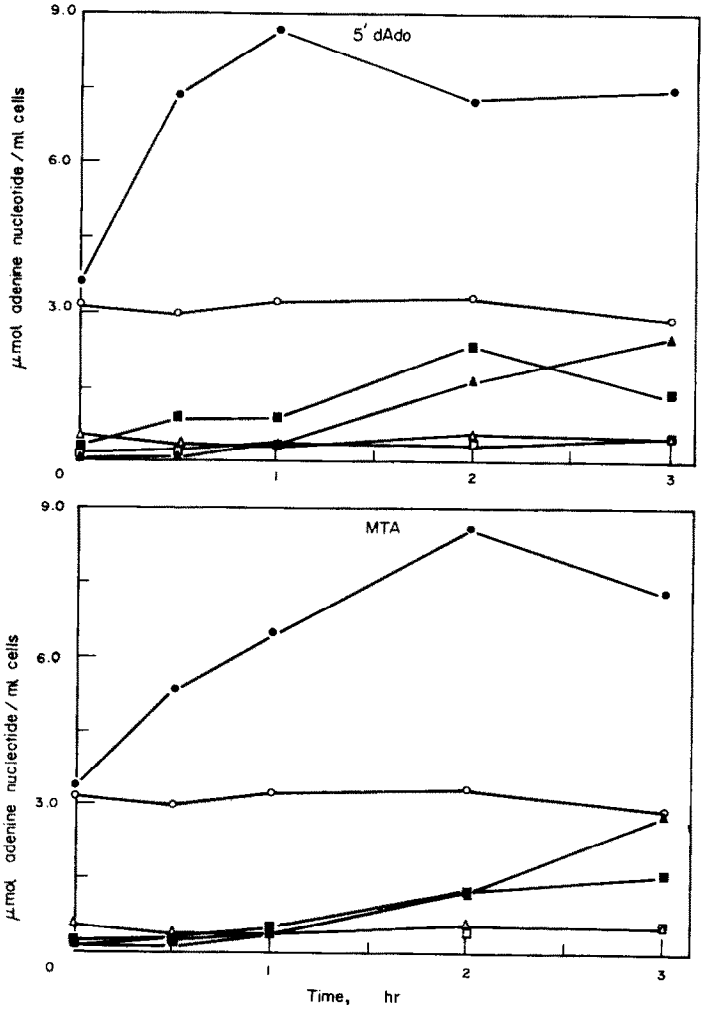

Fig. 6. Effects of 500 μM 5'-dAdo (upper panel) and 500 μM MTA (lower panel) on adenine nucleotide levels of Sarcoma 180 cells *in vitro*. Cells were obtained from mice 6–7 days after i.p. inoculation with approximately 2.5×10^6 cells. After several washes in saline, the cells were resuspended in 50 mM potassium phosphate (pH 7.4), containing 2 mM $MgSO_4$, 75 mM NaCl, 10 mM glucose, and 1 μg/ml dCF, at a final cell concentration of 3%, and were incubated in a shaking water bath at 37° for 20 min. The cells were then centrifuged and readjusted to a 3% suspension, in the above medium containing either 5'-dAdo or MTA (without dCF), and returned to the water bath. At the indicated times, aliquots were prepared for anion-exchange h.p.l.c. as outlined in Materials and Methods. Adenine nucleotide levels were determined by an anion-exchange h.p.l.c. technique reported previously [20]. Controls: AMP (△), ADP (□), and ATP (○); nucleoside-treated: AMP (▲), ADP (■), and ATP (●).

incubation of Sarcoma 180 cells with high levels of orthophosphate and glucose markedly increased steady-state levels of PRPP. Typically, in Sarcoma 180 cells, PRPP levels increased about 10-fold, from approximately 0.15 to 1.5–2.0 μmole/ml of cells, after 1 hr of incubation. Under similar conditions, 5′-dAdo inhibits these increases in PRPP levels in Ehrlich ascites cells but does not inhibit partially purified PRPP synthetase isolated from these cells [2].

The effects of 5′-dAdo, MTA, and adenine on PRPP accumulation in Sarcoma 180 cells are compared in Fig. 7. Each of these compounds inhibited PRPP accumulation in a dose-dependent manner, with 5′-dAdo the most effective (ID_{50} = 23 μM), adenine the next most effective (ID_{50} = 37 μM), and MTA the least effective inhibitor (ID_{50} = 50 μM). At concentrations of 5′-dAdo of 200 μM or more, PRPP was not detectable (< 0.02 μmole/ml of cells) after 1 hr of incubation. In fact, 5′-dAdo is the most potent inhibitor of PRPP accumulation among a number of adenosine analogs tested to date in this system [33].

DISCUSSION

These studies demonstrate that MTA phosphorylase is present in Sarcoma 180 cells, and that this enzyme reacts with 5′-dAdo, yielding adenine and 5-deoxyribose-l-phosphate. In cells treated with 5′-dAdo, a rapid increase in adenine nucleotide levels, especially ATP, was observed, as well as a dose-dependent inhibition of PRPP accumulation.

These data suggest a hypothesis for the mechanism by which 5′-dAdo causes the metabolic alterations reported previously [2]: 5′-dAdo is transported into the cell via nucleoside transport systems and is then phosphorolytically cleaved by cytoplasmic MTA phosphorylase to adenine and 5-deoxyribose-l-phosphate. The liberation of free adenine results in an increased utilization of intracellular PRPP, as the adenine is salvaged by APRT forming AMP. Further metabolism of AMP results in increases in the adenine nucleotides (see Fig. 6). Since ADP [34, 35] and possibly ATP [36] are inhibitory regulators of PRPP synthetase, their accumulation may restrict PRPP synthesis. In addition, the other product of the reaction of 5′-dAdo with MTA phosphorylase, 5-deoxyribose-l-phosphate, may inhibit phosphoribomutase. Because this enzyme is necessary for the production of PRPP from ribose-l-phosphate, inhibition of its activity could further diminish PRPP availability. Recent preliminary studies (E. Chu, unpublished observations) indicate that 5-deoxyribose-l-phosphate inhibits rabbit muscle phosphoglucomutase (believed to be responsible for phosphoribomutase activity as well), with a K_i value (75 μM) below the K_m of ribose-l-phosphate (170 μM [37]). Thus, 5′-dAdo cleavage would induce both an increased utilization of PRPP and a blockade of PRPP synthesis, accounting for the marked inhibition of PRPP accumulation observed in Sarcoma 180 (Fig. 7) and Ehrlich ascites cells [2]. The decreased availability of intracellular PRPP that occurs as a consequence of 5′-dAdo (and MTA) metabolism may explain why the rate of incorporation of 5′-dAdo (and MTA) into adenine nucleotide pools of Sarcoma 180 cells (Fig. 6) reaches a plateau following an initial rapid rate of incorporation, i.e. as ADP and ATP accumulate, the PRPP available for continuing nucleotide synthesis declines until no further adenine salvage occurs.

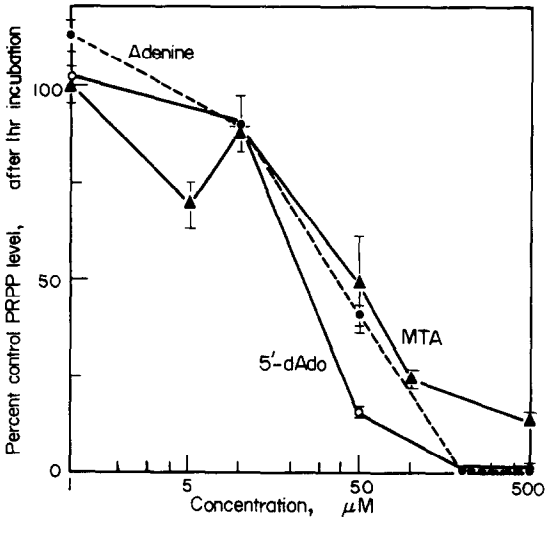

Fig. 7. Log dose–response curves of the effects of 5′-dAdo (○), MTA (▲), and adenine (●) on the accumulation of PRPP in Sarcoma 180 cells. Cells were obtained and prepared as described in Fig. 6, without the dCF pretreatment step. The cells (3% suspensions) were incubated with the indicated concentrations of either 5′-dAdo, MTA, or adenine and placed in a shaking water bath (37°). After 1 hr, the PRPP levels were determined by the method of Reem [25]. The control PRPP level after 1 hr was 1.98 ± 0.15 μmole/ml of cells. Data points represent the average of two determinations; bars indicate the range of values.

The reduced availability of PRPP could also explain the previously observed [2] 5′-dAdo-mediated inhibition of purine biosynthesis *de novo*, a PRPP-dependent process. Furthermore, elevations of AMP (see Fig. 6), a known feedback inhibitor of the rate-limiting enzyme of purine *de novo* synthesis, PRPP amidotransferase [38], may also limit purine synthesis. One may predict that the 5′-dAdo-induced depletion of PRPP levels would also impair other PRPP-dependent processes, such as (1) pyrimidine biosynthesis *de novo*, (2) the hypoxanthine–guanine phosphoribosyltransferase reaction, which would affect the salvage of hypoxanthine and guanine as well as the activation of drugs such as 6-mercaptopurine, and (3) the formation of 5-fluorouracil nucleotides via orotate phosphoribosyltransferase.

Finally, the 5′-dAdo-induced increases in ATP concentrations may account for the "crossover" at the phosphofructokinase step observed in the Ehrlich tumor system [2], ATP being a well-established regulator of this enzyme [39]. In short, the accumulation of the products of the reaction of 5′-dAdo with MTA phosphorylase, and/or their subsequent metabolism, can explain virtually all the effects of 5′-dAdo that had been ascribed to the unmetabolized nucleoside [2]. A summary of some of the postulated mechanisms of 5′-dAdo-induced metabolic alterations is presented in Fig. 8.

From this hypothesis, it can also be predicted that

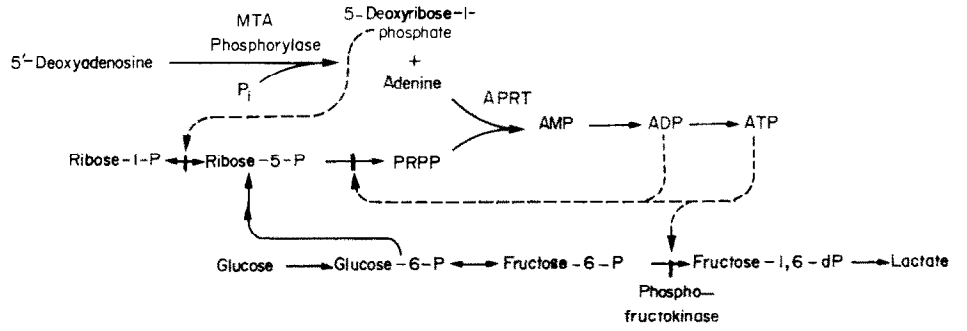

Fig. 8. Proposed mechanism of some of the metabolic alterations produced by 5'-dAdo. Inhibitory effects are represented by dotted lines.

adenine would mimic many of the effects of 5'-dAdo. Adenine is known to cause elevations of adenine nucleotides [36], to depress PRPP accumulation [40], and to inhibit purine *de novo* synthesis [41]. As shown in Fig. 7, both adenine and 5'-dAdo markedly depress PRPP accumulation in a dose-dependent manner; however, 5'-dAdo is more effective than adenine, suggesting that 5-deoxyribose-1-phosphate may play an important role; transport effects might also explain this difference.

The metabolism of 5'-dAdo may not be limited to its reaction with MTA phosphorylase. Palmer and Abeles [42] have reported that the *S*-adenosylhomocysteine hydrolase from beef liver reacts with 5'-dAdo, yielding 3'-keto-5'-deoxyadenosine. The role of this interaction, or of this metabolite, in the effects of 5'-dAdo has not yet been studied. Nevertheless, it is still possible that 5'-dAdo itself possesses as yet unidentified biological activities. Cell lines lacking MTA phosphorylase activity [11] could be of use in approaching this question.

The relative V_{max} of 5'-dAdo with MTA phosphorylase is about 1.8-fold higher than with the natural substrate, MTA. The reason for this faster turnover rate is unknown, but one may speculate that the rate-limiting step of the reaction mechanism (believed to follow an ordered bi–bi sequence [10]) is the release of the sugar phosphate from the enzyme. If the rate constant for the release of 5-methylthioribose-1-phosphate is appropriately lower than that for 5-deoxyribose-1-phosphate, the difference in the relative V_{max} values of the two substrates might be explained. In any case, as can be seen in Fig. 6, the initial rate of total adenine nucleotide formation (i.e. during the first 30 min) in Sarcoma 180 cells treated with 500 μM 5'-dAdo is more than twice that of cells treated with 500 μM MTA. The faster rate of nucleotide formation observed with 5'-dAdo could reflect the nearly 2-fold higher relative V_{max} of MTA phosphorylase with 5'-dAdo as compared to MTA. This implies that the MTA phosphorylase reaction is the rate-limiting step in the pathway for the synthesis of nucleotides from 5'-dAdo or MTA (see Fig. 8), although other explanations, such as differences in transport, cannot be ruled out. This 2-fold difference in V_{max} values may also explain, in part, the 2-fold lower ID_{50} value of 5'-dAdo for the inhibition of PRPP accumulation as seen in Fig. 7.

5'-dAdo has been proposed as a "nonmetabolized" probe for studying the nucleoside transport system [43]. While 5'-dAdo has been successfully employed in transport studies with L1210 cells [43], it has been reported [11] and confirmed in our laboratory that cells of this line grown in culture lack MTA phosphorylase. Because MTA phosphorylase, by its reaction with 5'-dAdo, would drastically affect the apparent transport behavior of 5'-dAdo, it is obligatory that investigators evaluate the status of this enzyme in the cell line employed before selecting 5'-dAdo as a tool for transport studies.

5'-Deoxynucleosides, which cannot be directly phosphorylated, as a class may have greater biological activity than previously suspected. 5'-Deoxyinosine is an excellent substrate for human erythrocytic PNP, with K_m and V_{max} values comparable to those of the natural substrate, inosine [1]. 5'-Deoxy-5-fluorouridine has activity against a broad spectrum of mouse and rat tumors [44, 45]. Recently, this compound has been shown to be a substrate for uridine phosphorylases from mouse liver and various murine tumor cells (Ref. 46; J. Niedzwicki and S. Cha, personal communication), suggesting that the initial steps in the activation of this agent are cleavage to 5-fluorouracil, with subsequent conversion to 5-fluorouridine-5'-monophosphate by reaction with orotate phosphoribosyltransferase. These findings, along with the above studies on the reaction of 5'-dAdo with Sarcoma 180 MTA phosphorylase, suggest the generalization that mammalian nucleoside phosphorylases do not require 5'-hydroxylated substrates for activity. One may speculate that this reflects a basic similarity in the active sites of mammalian nucleoside phosphorylases. Since the phosphorylases for uridine and thymidine are separate enzymes [47], one wonders whether this generalization will extend to thymidine phosphorylase.

From the present studies it appears that the MTA phosphorylase from Sarcoma 180 cells does not possess exacting substrate specificity with regard to the 5' position. A more detailed study of the structure–activity relationships of MTA phosphorylase employing 5'-substituted analogs of both 5'-dAdo and MTA, as well as an evaluation of their potential

as antineoplastic and immunosuppressive agents, will be the subject of subsequent communications.

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