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# PURIFICATION AND CHARACTERIZATION OF 5′-DEOXY-5′-METHYLTHIOADENOSINE (MTA) PHOSPHORYLASE FROM HUMAN LIVER

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**Abstract**—5′-Methylthioadenosine phosphorylase was purified 8000-fold from human liver using a combination of affinity chromatography, chromatofocusing and gel filtration. A 25% overall yield was obtained. The specific activity of the final preparation was 40 μmol of 5′-methylthioadenosine cleaved per hr per mg of protein. The enzyme had an apparent molecular weight of 55,000 daltons, as determined by gel filtration on Superose 12 and Sephadex G-150, with a subunit molecular weight of 30,000 daltons, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The substrate specificity of the purified enzyme was studied in both the direction of nucleoside cleavage and nucleoside synthesis.

5′-Deoxy-5′-methylthioadenosine (MTA‡) phosphorylase (5′-deoxy-5′-methylthioadenosine:orthophosphate methylthioribosyltransferase, EC 2.4.2.28) catalyzes the phosphorolysis of MTA. This sulfur-containing nucleoside is formed from the decarboxylation of *S*-adenosylmethionine as a result of spermidine and spermine biosynthesis [1]. This reaction serves the dual purpose of recycling the reaction products, adenine and 5-methylthioribose 1-phosphate (MTR-1P), into adenine nucleotides [1] and methionine [2], respectively, and removing MTA, which has been shown to inhibit several enzymes [3–5] as well as cellular growth processes [6, 7]. Several research groups have explored ways to modulate MTA phosphorylase activity to inhibit proliferation of malignant cell lines [8–10]. Others have studied analogous enzymes present in pathogens to exploit substrate specificity differences for chemotherapy [11, 12]. Although considerable research has been done characterizing the enzyme from human placenta [13], human prostate [14], various cell lines [9, 15–18], and non-human tissues [19–22], little is known about the specificity of the enzyme from major human organs. In this paper we report the purification of MTA phosphorylase from human liver. The study of the substrate specificity of the enzyme with a wide variety of purines and purine analogs has been made possible by the development of an assay utilizing radioactive MTR-1P, the common substrate in these reactions.

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‡ Abbreviations: DTT, dithiothreitol; HEPES, *N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; MTA, 5′-deoxy-5′-methylthioadenosine; MTR-1P, 5-methylthioribose-1-phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and TCA, trichloroacetic acid.

## MATERIALS AND METHODS

### *Chemicals*

MTA, dithiothreitol (DTT), purine, 6-methylpurine, 5′-deoxyadenosine, 8-bromoadenine, 2-methyladenine, 2-aminoadenine, 4-aminopyrazolo[3,4-*d*]pyrimidine, 6-methylaminopurine, Sephacryl S-200, and Sephadex G-25 were purchased from the Sigma Chemical Co., St. Louis, MO. [*Methyl*-$^{14}$C]-adenosylmethionine (40 mCi/mmol) was obtained from ICN Radiochemicals, Irvine, CA. [*Methyl*-$^{14}$C]MTA was formed from [*methyl*-$^{14}$C]*S*-adenosylmethionine by the method of Schlenk and Ehninger [23] and purified by preparative thin-layer chromatography (TLC) using cellulose plates (250 μm Avicel F layer, Analtech, Newark, DL). [U-$^{14}$C]Adenine (273 mCi/mmol) was purchased from Amersham International, Amersham, U.K. Polybuffer Exchanger 94 (PBE94), polybuffer 96 and cyanogen bromide-activated Sepharose 4B were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. A Dynamax C-18 HPLC column (1 cm i.d. × 25 cm) was obtained from Rainin Instruments, Woburn, MA. Xanthine oxidase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. 3-Methyladenine, 5′-tosyladenosine, 1-methyladenine, 1,3-phenylenediamine and 4-aminopyrimidine were from the Aldrich Chemical Co., Milwaukee, WI. PEI-cellulose TLC plates were manufactured by E. Merck, Darmstadt, Federal Republic of Germany. 5′-*O*-Methyladenosine was synthesized by the reaction of 5′-tosyladenosine with sodium methoxide and purified by reverse phase HPLC as described herein. The following compounds were synthesized in these laboratories and were shown to be greater than 99% pure by high-performance liquid chromatography: 4-aminopyrazolo[3, 4-*d*]pyrimidine 5′-deoxyriboside, 2-fluoroadenine, 1-deazaadenine, 3-deazaadenine and 7-deazaadenine. 5′-Deoxy-5′-methylthio-3-deazaadenosine was the gift of Dr. John Montgomery, Southern Research Institute, Birmingham, AL. Scintilene and Scintiverse liquid scintillation solutions

were purchased from the Fisher Scientific Co., Raleigh, NC. All other chemicals used were of the highest purity available.

*Protein determination*

Protein was quantitated by the method of Bradford [24] using Coomassie reagent and bovine serum albumin standard purchased from the Pierce Chemical Co., Rockford, IL.

*MTA phosphorylase assay*

Three assays were employed to measure MTA phosphorylase activity. All reactions were conducted at 37°.

For monitoring column fractions, a modification of the spectrophotometric assay described by Savarese *et al.* [16] was used. Reaction mixtures (500 μL) contained 50 mM potassium phosphate (pH 7.4), 0.2 mM MTA, 1 mM DTT, 0.8 I.U. xanthine oxidase and various amounts of MTA phosphorylase. The xanthine oxidase mediated oxidation of the liberated adenine was monitored at 305 nm. This assay was linear up to an absorbance change of 0.01 per min, corresponding to a rate of 0.02 μmol of MTA cleaved/hr.

For kinetic studies of nucleoside cleavage, a modification of the radiometric assay described by Seidenfeld *et al.* [25] was employed. Reaction mixtures (100 μL) contained 50 mM potassium phosphate (pH 7.4), 0.2 mM [*methyl*-$^{14}$C]MTA ($5 \times 10^3$ cpm/nmol), 1 mM DTT and various amounts of MTA phosphorylase. After a 30-min reaction period, 25 μL of 1 M trichloroacetic acid (TCA) and 200 μL of a 1:1 slurry of Dowex-50 in 0.2 M TCA were added to the reaction mixture. The tubes were agitated for 5 min and centrifuged briefly. An aliquant (180 μL) of the supernatant was added to a scintillation vial and an additional 100 μL of 0.2 M TCA was added to the assay tube containing the Dowex-50. The tube was vortexed and recentrifuged, and 100 μL of the supernatant was added to the 180 μL from the first centrifugation. Five milliliters of Scintiverse II was added to each vial and the radioactivity determined by liquid scintillation. This method accurately reflected the percent of substrate converted to product as confirmed by TLC separation (PEI-cellulose developed in 10 mM $NH_4HCO_3$, see below) of aliquots of reaction mixtures subjected to the Dowex-50 separation method.

For kinetic studies in the nucleoside synthesis direction, reaction mixtures (25 μL) contained 50 mM potassium HEPES (pH 7.0), 2.0 mM DTT, 0.4 mM [*methyl*-$^{14}$C]MTR-1P ($3.7 \times 10^3$ cpm/nmol, see below), 0.004 to 0.016 unit MTA phosphorylase (1 unit = 1 μmol MTA cleaved/hr) and various concentrations of adenine and/or purine analogs. Preliminary testing of nucleobases was performed at the highest attainable concentration in order to convert as much MTR-1P to the corresponding nucleoside as possible. A separate reaction was set up with 200 μM adenine as a positive control and as a TLC-mobility standard. MTA had a relative mobility of 0.47 in this system. After development of the TLC plate, the novel nucleoside was located using a Berthold LB 2760 TLC plate scanner. This information was used subsequently for $K_m$ determinations to provide a guide to cut out the appropriate area of the TLC plate. For determination of kinetic constants the reaction mixture was preincubated at 37° and the reaction started by addition of enzyme. After an appropriate time period, a 5-μL aliquot of the reaction mixture was spotted onto a PEI-cellulose TLC plate which had been prespotted with 15 nmol of MTA. The plate was immediately dried and developed with 10 mM $NH_4HCO_3$. The nucleoside region was cut out and placed in a scintillation vial. Ten milliliters of Scintilene was added to the vial and the radioactivity was determined by liquid scintillation.

*Test of potential activators and inactivators of MTA phosphorylase*

Undiluted enzyme was incubated with the potential activator or inactivator for 30 min at 37° in 50 mM potassium phosphate. DTT (1 mM) was included in tests of compounds that were not potential sulfhydryl modification reagents. The treated enzyme was assayed spectrophotometrically and the rate compared to that of control enzyme. If the compound being tested could potentially interfere with this assay procedure, the enzyme was freed of the compound by rapid desalting through Sephadex G-25 [26] prior to dilution into the assay reaction. No preincubation was used in testing potential physiological modulators of MTA phosphorylase activity. These compounds were incorporated into spectrophotometric assay mixtures containing MTA phosphorylase, and the resulting rate was compared to that of a control reaction in the absence of the compound.

*Purification procedure*

Human livers were obtained from the morgue 12–18 hr post-autopsy. The tissue used was from patients who had died of vascular or pulmonary disease and the liver was examined by the duty pathologist for any indication of liver pathology. Only normal livers were used. The liver samples were stored frozen at −80° until use.

*Homogenate and extract.* The frozen liver sections were broken into approximately 2.5 $cm^3$ pieces and placed in room temperature deionized water (350 g tissue/L). They were then stirred at room temperature for 30 min at which time glacial acetic acid was added to a concentration of 10 mM, solid DTT to a final concentration of 5 mM, and phenylmethylsulfonyl fluoride (100 mM in isopropanol) to a final concentration of 0.2 mM. This material was homogenized in a Waring blender (model CB-4) at 5°. All subsequent operations were carried out at this temperature unless otherwise specified. The blender was operated for 30 sec on low setting, 30 sec on medium setting and 2 min on high setting. The pH of the resulting homogenate was maintained at 5.5 with glacial acetic acid when necessary. The homogenate was centrifuged at 8000 *g* for 1 hr. The supernatant was passed through a funnel with a glass wool plug, and the pH of the filtrate was adjusted to 7.5 with 5 N KOH.

*Ammonium sulfate precipitation.* The supernatant was placed on ice, stirred and brought to 80%

$(NH_4)_2SO_4$ saturation by the gradual addition of solid $(NH_4)_2SO_4$ (516 g/L of supernatant). Stirring was continued for 30 min after all of the $(NH_4)_2SO_4$ had dissolved. The precipitate was collected by centrifugation at 8000 *g* and then dissolved in a minimal volume of Buffer A (50 mM potassium phosphate, pH 7.4, containing 1 mM DTT and 0.5 mM EDTA). The dissolved precipitate was dialyzed for 24 hr against four changes of 4 L of Buffer A. The dialyzed material was centrifuged at 15,000 *g* for 30 min, and the resulting supernatant was dispersed into 20-mL aliquots and stored at −20°.

*Affinity chromatography.* 6-(*p*-Aminobenzylamino) purine agarose was prepared in these laboratories by Dr. Lowrie Beacham, III, as described by Holguin and Cardinaud [27]. Twenty milliliters of liver extract was applied by gravity flow to a 2.5 × 14 cm column of this resin which had been equilibrated with Buffer A. The column was washed with 10 column volumes of Buffer A containing 0.5 M KCl. MTA phosphorylase was then eluted with 3 M urea in Buffer A and dialyzed for 24 hr against three changes of 100 volumes of Buffer A. The enzyme was then concentrated 10-fold in an Amicon concentration cell using a PM-10 membrane. Addition of KCl to 0.1 M while maintaining the DTT concentration at 1 mM improved the recovery of MTA phosphorylase activity during this ultrafiltration step.

*Chromatofocusing.* The ultrafiltration concentrate (15 mL) from the previous step was dialyzed against two changes of 100 volumes of 25 mM Tris–acetate (pH 8.3 at 5°) containing 1 mM DTT for 18 hr. The dialysate was applied in 5-mL aliquots with alternating 5-mL portions of Polybuffer 96 (diluted 1:13, adjusted to pH 6.0 with acetic acid) containing 1 mM DTT to a 1.5 cm × 27 cm PBE 94 column equilibrated with the same Tris–acetate buffer. Once all the sample was applied, the enzyme was eluted with the diluted Polybuffer. Fractions containing enzyme activity were pooled and brought to 50 mM potassium phosphate, 0.1 M KCl and 1 mM DTT by the addition of 1 M potassium phosphate buffer (pH 7.4), 2 M KCl and 0.5 M DTT, respectively. The resulting enzyme solution was concentrated by ultrafiltration as described above.

*Sephacryl S-200 chromatography.* The concentrated material obtained from chromatofocusing (5–7 mL) was applied by gravity flow to a 2.5 × 43 cm column of Sephacryl S-200 equilibrated with Buffer A containing 0.1 M KCl. After sample loading, the column was washed at a flow rate of 1 mL/min with Buffer A. Fractions containing MTA phosphorylase activity were pooled and concentrated by ultrafiltration as previously described. Then the enzyme solution either was stored at −70° or, if a phosphate-free preparation was desired, it was dialyzed for 24 hr against three changes of 200 volumes of 50 mM HEPES, pH 7.0, containing 1 mM DTT and 0.5 mM EDTA and then stored at −70°.

*Polyacrylamide gel electrophoresis*

Native electrophoresis of protein samples was performed in slab gels with a 7.5% separating gel containing 2 mM DTT and a 2.5% stacking gel photopolymerized with riboflavin-phosphate according to the method of Davis [28]. MTA phosphorylase was detected in gel slices by incubation with 0.5 mL of the spectrophotometric assay mixture lacking xanthine oxidase. After an overnight incubation with gentle shaking at 37°, the liquid was removed from the slices and placed in a cuvette thermostated at the same temperature. The adenine formed in the course of the overnight incubation was determined by addition of xanthine oxidase and monitoring absorbance at 305 nm. Electroelution of protein from gel slices was performed using an Isco electroelution apparatus (Isco Instruments, Lincoln, NE) using one of the recommended buffer systems (Tris–acetate plus 1 mM DTT).

Electrophoresis under denaturing conditions was performed using 10% separating gels according to the method of Laemmli [29]. When it was desirable to perform SDS–PAGE on a sample with a low protein concentration, equal volumes of the sample and 10% TCA were mixed and incubated on ice for 30 min. The sample was then centrifuged at 5° for 30 min at 30,000 *g* and the supernatant carefully removed and discarded. Ice-cold acetone was added to the precipitate and the tube placed at −20° for 30 min followed by another centrifugation. The supernatant was discarded, the precipitated protein allowed to air dry at room temperature, and a small volume of SDS sample buffer (62.5 mM Tris–Cl, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.005% bromophenol blue) added.

*Synthesis of* [$^{14}$C]*MTR-1P*

This compound was enzymatically synthesized using partially purified human liver MTA phosphorylase (purified at least through the affinity chromatography step) and [*methyl*-$^{14}$C]MTA. A typical reaction contained 0.2 mM MTA, 1 mM DTT, $1.6 \times 10^6$ cpm [*methyl*-$^{14}$C]MTA (40 mCi/mmol), 50 mM potassium phosphate (pH 7.4), and 0.2 unit MTA phosphorylase per mL of reaction. This mixture was incubated for 1.5 to 2.0 hr at 37° and then brought to 10 mM $NH_4HCO_3$ by addition of 1 M $NH_4HCO_3$. The resulting solution was loaded onto a DEAE-Sephadex A-25 column equilibrated with 25 mM $NH_4HCO_3$. The column was washed with the same buffer until unbound radioactivity was completely eluted. The MTR-1P was eluted with a gradient (10 column volumes) from 25 to 500 mM $NH_4HCO_3$. A single peak of radioactivity was collected and lyophilized to reduce the concentration of $NH_4HCO_3$. The lyophilized sample was dissolved in a small volume of ice-cold water, adjusted to pH 4.0 with acetic acid and purified by reverse-phase HPLC on a C-18 column (1 cm × 25 cm) equilibrated with water. Elution of this column with water at a flow rate of 4.0 mL/min resolved MTR-1P from inorganic phosphate [11]. Inorganic phosphate was quantitatively monitored by the method of Chen *et al.* [30].

*Synthesis of 5′-O-methyladenosine*

This compound was produced by the reaction between 5′-tosyladenosine and sodium methoxide, as described. Sodium metal (0.29 g; 13 mmol) was

Table 1. Purification of MTA phosphorylase

| Fraction | Protein (mg) | Units/mg* | Fold purification | % Recovery |
|---|---|---|---|---|
| Homogenate | 260,000 | 0.0048 | 1.0 | 100 |
| Extract | 180,000 | 0.0062 | 1.3 | 91 |
| $(NH_4)_2SO_4$ ppt. | 16,000 | 0.062 | 13 | 83 |
| Affinity chrom. | 790 | 0.81 | 169 | 54 |
| Chromatofocusing | 460 | 8.1 | 1690 | 32 |
| Sephacryl S-200 | 7.6 | 40.5 | 8450 | 25 |

* One unit of enzyme will cleave 1 μmol MTA/hr.

added to 100 mL anhydrous methanol in a round bottom flask fitted with a drying tube. After the sodium had reacted completely, 0.54 g (1.3 mmol) 5′-tosyladenosine was added and the flask fitted with a water-jacketed condenser. The reaction was refluxed for 18 hr at which time TLC indicated complete loss of the starting material. The methanol was removed *in vacuo*. The remaining solid was dissolved in 50 mL water and neutralized with acetic acid. The neutralized sample was applied to a 4.5 × 22 cm column of Lichroprep RP-C18, 40–63 μm (E. Merck) equilibrated with 10% acetonitrile in water and the column washed with this same solvent. 5′-*O*-Methyladenosine was obtained in 30% theoretical yield with a purity of 95% as assessed by reverse-phase HPLC. The compound was further purified (>98%) by HPLC on a C-18 reverse-phase column. The proton nuclear magnetic resonance and ultraviolet spectra were identical to those reported for 5′-*O*-methyladenosine [31].

*Determination of kinetic constants*

All velocities were calculated from initial linear rates. Reaction velocities from radiochemical assays were determined from time course experiments from the slope of a plot of product formed versus time. Kinetic constants were determined by directly fitting the data to a hyperbola according to the method of Wilkinson [32] by the use of the computer program of Cleland [33]. Enzyme inhibition was analyzed according to the method of Spector and Hajian [34].

## RESULTS

*Purification of MTA phosphorylase from human liver*

MTA phosphorylase was purified according to the sequence of procedures outlined in Table 1. An 8000-fold purification and an overall yield of 25% was obtained. The final preparation contained no detectable purine nucleoside phosphorylase activity (<0.2%) or adenosine deaminase activity (<0.3%). Activity measurements of the bands on non-denaturing gels indicated that the purified enzyme was about 5–15% pure. The calculated specific activity of homogeneous enzyme should therefore be 4.5 to 13.4 μmol/min/mg protein, a value essentially the same as that reported for the homogeneous enzyme from human placenta [13] and beef liver [22].

Several of the chromatographic procedures previously described for the purification of MTA phosphorylase from human placenta [13] were tried with the human liver but were not as effective in our hands. Virtually homogeneous MTA phosphorylase could be obtained via the procedure described in the present paper by extensive washing of the 6-(aminobenzylamino)purine agarose column, reversing the order of the gel filtration and chromatofocusing steps and by using the FPLC Mono-P column instead of chromatofocusing but the yield was only 7%. Also, this procedure required a final dialysis step to remove Polybuffer from the enzyme.

*Physical characterization of human liver MTA phosphorylase*

Gel filtration of both crude and partially purified preparations of the enzyme on calibrated Superose 12 columns with the FPLC system yielded identical values for the apparent molecular weight of 55,000 daltons.

Nondenaturing polyacrylamide gel electrophoresis of MTA phosphorylase followed by assay of gel slices yielded a relative mobility of 0.4 (data not shown). Electroelution of this region of the gel allowed recovery of active MTA phosphorylase which, after TCA precipitation and SDS–PAGE, revealed a single silver-stained band of 30,000 daltons confirming the subunit identity and molecular weight. This value is in good agreement with the value of 32,500 daltons reported for the subunit molecular weight of human placental MTA phosphorylase [13].

*Catalytic characterization of human liver MTA phosphorylase*

The enzyme activity, as measured by MTA cleavage, was dependent upon the presence of phosphate in the assay. A 6-fold increase in rate was observed upon addition of phosphate (50 mM) to an essentially "phosphate free" spectrophotometric assay mix (50 mM HEPES buffer). In the absence of DTT, enzyme activity was reduced approximately 50%; addition of DTT to a concentration of 1 mM caused activity to return to approximately 75% of the original level.

*Specificity of nucleoside synthesis*

To obtain data on the substrate specificity of human liver MTA phosphorylase with regard to the nucleobase moiety, an assay in the direction of

Table 2. Nucleobase specificity of MTA phosphorylase

| Nucleobase substrate | $K_m$* (μM) | $K_i$* (μM) | Relative $V_{max}$† |
|---|---|---|---|
| Adenine | 20 ± 2.7 (8) | | 100 |
| 1-Methyladenine | | >3000 | <5 |
| 2-Methyladenine | 32 ± 8 (2) | | 122 |
| 2,6-Diaminopurine | 13 ± 0.2 (2) | | 65 |
| 2-Hydroxyadenine | 350 ± 70 (2) | | 43 |
| 2-Fluoroadenine | 17 ± 2.6 (3) | | 25 |
| 3-Methyladenine | | >3000 | <5 |
| 8-Bromoadenine | | >60 | <5 |
| 6-Methylaminopurine | 80 ± 4 (2) | | 6 |
| 6-Anilinopurine | | >140 | <5 |
| Purine | 140 ± 7(2) | | 8 |
| 2-Aminopurine | | >3000 | <5 |
| Hypoxanthine | | >3000 | <5 |
| 6-Mercaptopurine | | >450 | <5 |
| 6-Methylmercaptopurine | | >1600 | <5 |
| 6-Chloropurine | | >660 | <5 |
| 6-Methylpurine | 190 ± 9 (2) | | 8 |
| 1-Deazaadenine | 29 ± 0.4 (2) | | 220 |
| 3-Deazaadenine | | 22 ± 4 (2) | <5 |
| 7-Deazaadenine | | 300 ± 50 (2) | <5 |
| 8-Azaadenine | 11 ± 0.9 (2) | | 5 |
| 4-Aminopyrazolo [3,4-*d*] pyrimidine | | >3000 | <5 |
| Cytosine | | >6000 | <5 |
| 4-Aminopyrimidine | | >6000 | <5 |
| 5-Methylthioribose-1-phosphate | 27 ± 3 (4) | | |

* Values are the means ± SEM of (N) determinations. All of the values were determined using five velocity measurements over a 9-fold range of substrate concentration that spanned the $K_m$ value. Inhibition constants were determined using two inhibitor concentrations. In all cases, the intraexperimental error and the interexperimental error were comparable.

† Less than values represent the lower limit of detectability of the compound serving as substrate at a concentration of 1 mM.

glycosidic bond synthesis was developed. Development of this assay obviates the unavailability of 5′-deoxy-5′-methylthionucleosides and other appropriately 5′-modified nucleosides. Della Ragione *et al.* [13] have reported that the equilibrium of the reaction catalyzed by MTA phosphorylase lies far in the direction of nucleoside synthesis ($K_{eq} = 1.39 \times 10^{-2}$ in the direction of phosphorolysis, at 37° and pH 7.4) thus facilitating this assay method. The advantage of this assay is that by using [*methyl*-$^{14}$C]MTR-1P, any nucleobase can be examined for either substrate activity or inhibitory activity of [*methyl*-$^{14}$C]MTA synthesis. The assay was linear with respect to time and enzyme concentration up to approximately 30% conversion of MTR-1P to MTA under the conditions described. When the assay was used for determination of kinetic constants, the substrate conversion never exceeded 20% of either substrate. Through the use of this method, the data presented in Table 2 were generated. At a saturating adenine concentration (600 μM) the $K_m$ for MTR-1P was 27 ± 3 μM.

*Specificity of nucleoside phosphorolysis*

The xanthine oxidase coupled assay method is generally applicable to all nucleosides containing adenine or another nucleobase moiety which is a substrate for xanthine oxidase. A comparison of the $K_m$ values for MTA in the spectrophotometric assay and the radiochemical assay, 3 ± 1 and 2 ± 1 μM, respectively, validated both assay procedures. Using the spectral assay, the kinetic constants shown in Table 3 were determined. At a saturating MTA concentration (50 μM) the $K_m$ for phosphate was 580 ± 90 μM.

*Effect of small molecules on MTA phosphorylase activity*

Several small molecules were tested as potential regulators of MTA phosphorlytic activity; none had any effect. The compounds and the concentration at which they were tested by the spectrophotometric assay are as follows putrescine (1 mM), spermidine (1 mM), spermine (1 mM), and *S*-adenosylhomocysteine (1 mM). Compounds that were tested as potential inactivators of MTA phosphorylase but had no effect were periodate-oxidized MTA (200 μM), periodate-oxidized 5′-deoxyadenosine (200 μM), 6-chloropurine (1 mM), 8-bromoadenine (200 μM) and 6-[(1-methyl-4-nitroimidazol-5-yl)-thio]purine (1 mM).

Table 3. Nucleoside specificity of MTA phosphorylase

| Nucleoside substrate | $K_m$* ($\mu$M) | $K_i$* ($\mu$M) | Relative $V_{max}$† |
|---|---|---|---|
| 5′-Deoxy-5′-methylthioadenosine | 1.5 ± 0.2 (8) | | 100 |
| 5′-*O*-Methyladenosine | 5.5 ± 0.5 (2) | | 120 |
| 5′-Chloro-5′-deoxyadenosine | 1.2 ± 0.1 (2) | | 33 |
| Adenosine | 760 ± 90 (3) | | 62 |
| 2′-Deoxyadenosine | | >5400 | <3 |
| 5′-Deoxyadenosine | 3.1 ± 0.3 (4) | | 100 |
| 2′,3′-Dideoxyadenosine | | >500 | <3 |
| 2′,5′-Dideoxyadenosine | 9 ± 1 (2) | | 40 |
| Inosine | | >500 | <3 |
| 5′-Deoxy-5′-methylthio-3-deazaadenosine | | 60 ± 15 (2) | <3 |
| 4-Aminopyrazolo[3,4-*d*]pyrimidine 5′-deoxyriboside | | 167 ± 19 (2) | <3 |
| Phosphate | 580 ± 90 (4) | | |

* Values are the means ± SEM of (N) determinations. All of the values were determined using five velocity measurements over a 9-fold range of substrate concentration that spanned the $K_m$ value. Inhibition constants were determined using two inhibitor concentrations. In all cases, the intraexperimental error and the interexperimental error were comparable.

† Less than values represent the lower limit of detectability of the compound serving as substrate at a concentration of 1 mM.

## DISCUSSION

Our interest in nucleoside metabolism prompted us to initiate the work described in this paper. A survey of the literature on MTA phosphorylase reveals a variety of physical and kinetic properties reported for it. In particular, different kinetic constants have been reported for the enzyme from different tissues of the same organism (i.e. there is wide variation in the $K_m$ values reported for MTA for the enzyme from rat lung [19], rat brain [21], and rat prostate [35] and similar differences between reported $K_m$ values for human placenta [13] and human prostate [14]). There also exists variation in the molecular weight of the enzyme from various mammalian tissues. Riscoe and Ferro [36] and Della Ragione *et al.* [22] report molecular weights of 90,000 and 98,000, respectively, for the enzyme from bovine liver, while Gillet *et al.* [37] obtained a value of 57,000 for the enzyme from the same tissue. The value of 55,000 obtained in this report is different from the value of 98,000 reported by Della Ragione *et al.* [13] for the enzyme from human placenta. The specific activity of the final fraction reported in this paper is approximately 15-fold less than that reported by Della Ragione *et al.* [13] for their homogeneous preparation from human placenta. While the kinetic constants for the normal substrates adenine, MTA and phosphate determined for human liver are all reasonably similar to those reported for the human placental enzyme, the difference in the determined molecular weight values, if valid, would lead to a conclusion that either tissue-specific enzyme forms exist or that tissue-specific post-translational modification has occurred.

The data in Table 2 reveal several generalizations regarding the substrate specificity of MTA phosphorylase from human liver. The conclusion that an amino group in the 6-position of the purine ring is not essential for binding activity but is required for optimal affinity can be drawn by comparing the $K_m$ for adenine (20 $\mu$M) to that of purine (140 $\mu$M). Similarly, small electron donating substituents in the 6-position were tolerated (6-methylpurine and 6-methylaminopurine) but bulky substituents (6-anilinopurine) or electron withdrawing substituents (hypoxanthine and 6-chloropurine) did not have any substrate or inhibitory activity. Alterations in the purine ring system itself were accepted with variable results. 1-Deazaadenine was a good substrate for the enzyme, while 8-azaadenine exhibited a low $K_m$ but a low $V_{max}$ value. 3-Deazaadenine showed good inhibition of enzyme activity with a $K_i$ value equivalent to the $K_m$ value for adenine, whereas 7-deazaadenine was neither a substrate nor a good inhibitor ($K_i$ = 300 $\mu$M). Human liver MTA phosphorylase had a strict specificity for a fused ring system, showing no activity or inhibition with 4-aminopyrimidine or cytosine. Interestingly, while the 5′-deoxyribonucleoside of 4-aminopyrazolo[3,4-*d*]pyrimidine was a competitive inhibitor ($K_i$ = 167 $\mu$M, Table 3), the aglycone, 4-aminopyrazolo[3,4-*d*]pyrimidine, was neither a substrate nor an inhibitor. The converse was observed with 3-deazaMTA in that the aglycone, 3-deazaadenine, was a good inhibitor of MTA synthesis but the nucleoside was not a substrate and its inhibitory activity was not as good as would be expected from the result obtained with 3-deazaadenine. This suggested that there may be a strong effect of nucleoside conformation in the binding of a nucleoside to the active site. Substituents in the 2-position of adenine were tolerated as evidenced by the substrate activity of the 2-amino, 2-methyl, 2-fluoro, and 2-hydroxy adenines. However, removal

of the 6-amino group from 2,6-diaminopurine resulted in loss of both substrate activity and binding as evidenced by the findings for 2-aminopurine. Methyl groups in the 1- or 3-position of adenine abolished both substrate and binding activity possibly due to steric interference, destruction of H-bonding sites, or promotion of the imino form of these compounds.

The results presented in Table 3 extend the observations of previous researchers [9, 38]. Polar substituents in the 5′-position dramatically reduced binding activity as can be seen by comparing $K_m$ values for MTA and adenosine. The 5′-methylthio group was not essential for binding or substrate activity as 5′-deoxyadenosine and 5′-deoxy-5′-chloroadenosine served as equally good substrates relative to MTA. The result obtained with 5′-methoxyadenosine is interesting in that it indicates that the enzyme does not discriminate against oxygen in the 5′-position as long as it is in the ether form and not present as a hydroxyl group.

Hopefully, these results will serve as a basis for extending our understanding of the role MTA phosphorylase plays in the catabolism of nucleosides and nucleoside analogs.

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