Biochemical Pharmacology, Vol. 44, No. 4, pp. 775–785, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

HUMAN KIDNEY THIOPURINE METHYLTRANSFERASE

PHOTOAFFINITY LABELING WITH *S*-ADENOSYL-L-METHIONINE

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(*Received* 13 *January* 1992; *accepted* 20 *April* 1992)

Abstract—Thiopurine methyltransferase (TPMT) catalyzes the S-methylation of heterocyclic and aromatic sulfhydryl compounds such as the thiopurine drug 6-mercaptopurine (6-MP). TPMT activity in human tissue is regulated by a common genetic polymorphism, and "pharmacogenetic" variation in TPMT activity is an important factor in individual differences in thiopurine drug metabolism, toxicity and therapeutic efficacy. Human renal tissue contains two isozymes of TPMT, Peak I and Peak II, that can be separated by ion exchange chromatography. Our experiments were performed to determine whether *S*-adenosyl-L-methionine (Ado-Met), the methyl donor for the TPMT reaction, could be used as a photoaffinity ligand for these isozymes as one step in the study of the molecular basis for the TPMT genetic polymorphism. When [^{3}H-*methyl*]Ado-Met and partially purified preparations of either isozyme of human kidney TPMT were exposed to ultraviolet light at 254 nm, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a 35 kDa protein was the predominant species that was radioactively labeled. The same 35 kDa protein was photoaffinity labeled with [^{14}C-*carboxyl*]Ado-Met, demonstrating that labeling involved covalent binding of Ado-Met rather than methylation of the protein. TPMT enzymatic activity co-eluted with the 35 kDa protein during sequential DEAE ion exchange, gel filtration and hydroxylapatite chromatography. Inhibitors of TPMT enzymatic activity including *S*-adenosyl-L-homocysteine, sinefungin, 6-methylmercaptopurine and 3,4-dimethoxy-5-hydroxybenzoic acid inhibited photoaffinity labeling of the 35 kDa protein in preparations of both TPMT Peak I and Peak II isozymes in a concentration-dependent fashion, as did 6-MP, the methyl acceptor substrate for the TPMT reaction. All of these results were compatible with the conclusion that the 35 kDa protein was TPMT. Photoaffinity labeling of TPMT with [^{3}H]Ado-Met should make it possible to purify the enzyme to homogeneity and to study amino acid sequences at or near its active site.

Thiopurine methyltransferase (TPMT†, EC2.1.1.67) is an *S*-adenosyl-L-methionine (Ado-Met)-dependent cytoplasmic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds including the thiopurine drug 6-mercaptopurine (6-MP) [1–3]. The level of TPMT activity in the human red blood cell (RBC) is controlled by a common genetic polymorphism [4], and levels of TPMT activity in the RBC reflect relative levels of the enzyme activity in other human cells and tissues including the kidney, liver and lymphocyte [5–7]. The TPMT genetic polymorphism represents a striking example of the clinical significance of "pharmacogenetic" variation in a drug-metabolizing enzyme activity. Inherited variation in TPMT activity is an important factor responsible for individual differences in thiopurine-induced toxicity and in the therapeutic efficacy of thiopurine drugs. Patients with genetically low levels of RBC TPMT activity who are treated with 6-MP or azathioprine have very high RBC concentrations of 6-thioguanine nucleotides (6-TGN), active metabolites of the parent thiopurine drugs [8–10]. These patients are at greatly increased risk for the development of thiopurine-induced myelosuppression [9, 11, 12]. Conversely, patients with high levels of enzyme activity may be undertreated with standard doses of 6-MP [10]. Indirect evidence also suggests that the TPMT genetic polymorphism may be a risk factor for the occurrence of hypoprothrombinemia in patients treated with cephalosporin antibiotics, such as moxalactam, that yield heterocyclic sulfhydryl metabolites that are capable of undergoing S-methylation catalyzed by TPMT [13].

The molecular basis for inherited variation in TPMT activity is unknown. Immunoprecipitation performed with rabbit polyclonal antibodies to partially purified human kidney TPMT demonstrated that subjects with genetically low levels of RBC and renal enzyme activities also have lower levels of immunoreactive TPMT protein than do individuals with higher levels of enzyme activity [5]. In addition, human renal tissue contains two isozymes of TPMT, Peak I and Peak II, that can be separated by DEAE ion exchange chromatography [14] (Fig. 1). Other than their behavior during ion exchange chromatography, no differences in the biochemical properties of these two isozymes have been detected, and the

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† Abbreviations: TPMT, thiopurine methyltransferase; Ado-Met, *S*-adenosyl-L-methionine; 6-MP, 6-mercaptopurine; RBC, red blood cell; 6-TGN, 6-thioguanine nucleotides; Ado-Hcy, *S*-adenosyl-L-homocysteine; DTT, dithiothreitol; DMHBA, 3,4-dimethoxy-5-hydroxybenzoic acid; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and BAP, "brightness area product".

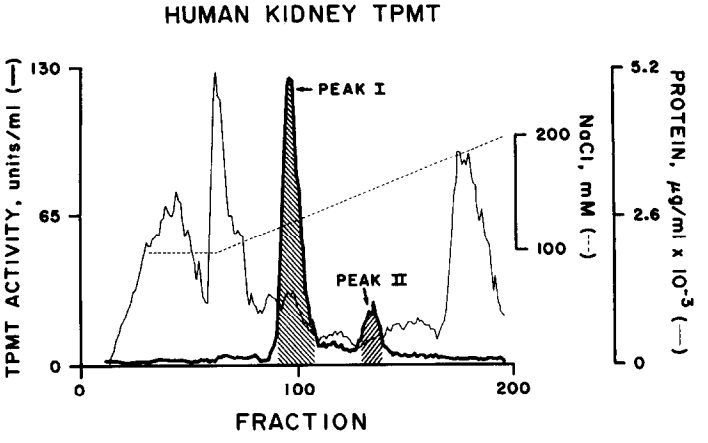

Fig. 1. DEAE ion exchange chromatography of human kidney TPMT. The two isozymes of TPMT are labeled as "Peak I" and "Peak II". Reproduced with permission from *Drug Metab Dispos* **18**: 632–638, 1990. Copyright (1990) the American Society for Pharmacology and Experimental Therapeutics. [Ref. 14].

existence of the isozymes does not appear to explain the molecular mechanism for the genetic polymorphism [14]. Ultimately, application of the techniques of molecular biology will be required to define the molecular basis for this polymorphism. However, attempts to apply those techniques have been complicated by difficulty in purifying the enzyme. TPMT loses catalytic activity when highly purified [2]. Development of a radioactive photoaffinity ligand for TPMT would allow the detection and subsequent purification of catalytically inactive enzyme protein. Ado-Met, the methyl donor for the reaction catalyzed by TPMT, has been used as a photoaffinity reagent for other methyltransferase enzymes [15, 16]. Our experiments were performed to determine whether Ado-Met might also serve as a photoaffinity ligand for the isozymes of human kidney TPMT. We found that a 35 kDa protein present in partially purified human kidney TPMT Peak I or Peak II preparations could be radioactively labeled by photoactivation of [^{3}H-*methyl*]Ado-Met, and that this labeling could be blocked in a concentration-dependent fashion by inhibitors of TPMT enzymatic activity. Therefore, the 35 kDa protein in both Peak I and Peak II preparations almost certainly represents TPMT. Photoaffinity labeling of TPMT with [^{3}H-*methyl*]Ado-Met should now make it possible to purify the enzyme to homogeneity and to determine amino acid sequences at or near its active site.

MATERIALS AND METHODS

Materials. [^{3}H-*methyl*]Ado-Met (sp. act. 72.5 to 78.4 Ci/mmol) and [^{14}C-*carboxyl*]Ado-Met (sp. act. 56.9 mCi/mmol) were obtained from DuPont-NEN, Boston, MA. [^{14}C-*methyl*]Ado-Met (sp. act. 48 mCi/mmol) was purchased from Research Products International, Mt. Prospect, IL. Ado-Met HCl, *S*-adenosyl-L-homocysteine HCl (Ado-Hcy), dithiothreitol (DTT), 6-MP, 6-methylmercaptopurine and 3,4-dimethoxy-5-hydroxybenzoic acid (DMHBA) were purchased from the Sigma Chemical Co., St. Louis, MO. Sinefungin and hydroxylapatite were obtained from Calbiochem, San Diego, CA. A low molecular weight protein market kit, Sephadex G-100 superfine, and DEAE CL-6B Sepharose were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Dye reagent for the protein assay was obtained from BioRad Laboratories, Richmond, CA. RESOLUTION® autoradiogram enhancer was purchased from the E.M. Corp., Chestnut Hill, MA.

Source of tissue. Renal tissue was obtained from patients undergoing clinically-indicated nephrectomies. All tissue was obtained under guidelines approved by the Mayo Clinic Institutional Review Board. Grossly normal tissue was placed immediately on dry ice and was stored at −80°. TPMT activity is stable in human tissue under these conditions of storage [2, 5, 14, 17].

TPMT assay. Renal TPMT activity was measured by the method of Weinshilboum *et al.* [18]. This assay is based on the conversion of 6-MP to radioactively labeled 6-methylmercaptopurine with [^{14}C-*methyl*]Ado-Met as the methyl donor. The final concentrations of 6-MP and Ado-Met, the substrates for the reaction, were 3.7 mM and 25 μM, respectively. The radioactively labeled reaction product was separated from the radioactive methyl donor by organic solvent extraction. One unit of enzyme activity represented the formation of 1 nmol 6-methylmercaptopurine/hr of incubation at 37°.

Protein assay. Protein concentrations were measured by the dye-binding method of Bradford [19] with bovine serum albumin (BSA) as a standard.

Partial purification of human kidney TPMT. The two isozymes of human kidney TPMT were separated and partially purified with a modification of the procedure described by Van Loon and Weinshilboum [14]. That procedure involves separation of the two isozymes by DEAE ion exchange chromatography (Fig. 1), followed by gel filtration chromatography

performed with Sephadex G-100 superfine. For some experiments, hydroxylapatite chromatography was added as a third chromatographic step. In those experiments, approximately 4 mg of protein purified through ion exchange and gel filtration chromatography was applied to a 1 × 20 cm hydroxylapatite column. Elution was performed with 20 mL of 5 mM potassium phosphate buffer, pH 7.5, that contained 1 mM DTT and 0.2 mM EDTA, followed by a 200 mL linear gradient from 5 to 75 mM potassium phosphate buffer, pH 7.5, that contained 1 mM DTT and 0.2 mM EDTA. The flow rate of the column was 0.5 mL/min, and 3.5-mL fractions were collected. Fractions that contained the highest TPMT activity were pooled and concentrated in an Amicon pressure concentrator with a YM-10 membrane, followed by centrifugation for 30 min at 5000 *g* in a Centricon-10 microconcentrator. The final preparation after hydroxylapatite chromatography was purified approximately 470-fold for Peak I, with a 2.8% yield, and approximately 89-fold for Peak II, with a yield of 0.14%. In both cases the data for purification and yield were calculated on the basis of comparison with total TPMT activity present in a 100,000 *g* supernatant. As discussed subsequently, estimates of the degree of purification of both enzymes, but especially those for Peak II, represent underestimates because of loss of enzyme activity during the purification procedure.

Photoaffinity labeling. The reaction mixture for photoaffinity labeling consisted of 20 μL of partially purified TPMT Peak I or Peak II (3–10 μg protein) in 20 mM Tris–HCl buffer, pH 7.5, that contained 1 mM DTT and 0.2 mM EDTA. To this mixture was added 5 μL of a solution of [^{3}H-*methyl*]Ado-Met (37 pmol, 2.75 μCi) to yield a final Ado-Met concentration of 1.2 μM. In some experiments [^{14}C-*carboxyl*]Ado-Met (1.75 pmol, 0.1 μCi) was used instead of [^{3}H-*methyl*]Ado-Met. During preliminary studies, the reaction mixture was placed in 12 × 75 mm siliconized glass culture tubes in a Rayonet model RPR-100 photochemical chamber. Photolysis was carried out at 4° with 254, 300 or 350 nm wavelength light sources with intensities of 8, 3.9 and 4.5 W, respectively, all located 6 cm from the tubes. After experiments performed with the Rayonet photochemical chamber had demonstrated that [^{3}H-*methyl*]Ado-Met could be used as a photoaffinity ligand for TPMT, subsequent experiments were performed with a stainless steel chamber designed to eliminate the absorption of ultraviolet (UV) light by glass, to allow easier handling of microliter reaction volumes, and to increase the number of samples that could be studied in a single experiment. In studies performed with the stainless steel chamber, the samples were placed in 96-well microtiter plates and were positioned 6 cm below a horizontal UV light source. When the microtiter plates were used, 76–86% of the total protein in each "well" was recovered after photolysis was performed. Immediately after photolysis, samples were transferred to polyethylene microcentrifuge tubes, and 25 μL of 0.25 M Tris that contained 40% glycerol, 20% 2-mercaptoethanol and 10% sodium dodecyl sulfate (SDS) was added to each sample. The samples were then heated at 95° for 5 min, and SDS–polyacrylamide gel electrophoresis (PAGE) was performed at 30 mA/gel in 1.5-mm thick 10% acrylamide slab gels with the discontinuous buffer system of Laemmli [20]. After electrophoresis, the gels were stained with 0.05% Coomassie Blue and were treated with the autoradiogram enhancer RESOLUTION® prior to drying. Autoradiograms were then obtained with Kodak X-Omat AR X-ray film. Exposure times for experiments performed with [^{3}H-*methyl*]Ado-Met varied from 1 to 30 days at −80°, while the exposure time for experiments performed with [^{14}C-*carboxyl*]Ado-Met was 12 weeks.

Analysis of autoradiograms. Autoradiograms were digitized with an 8 bit Micro Tek 300GS grey scale scanner and a Sun 3/160 workstation. Images were scanned at a resolution of 150 dpi, equivalent to 22,500 pixels per square inch. ANALYZE software [21] was used to quantitate "brightness area product" (BAP), i.e. the sum of intensities of all pixels within a defined region of the autoradiogram. Background BAP values were determined for each lane and were subtracted from BAP values for the region of the gel that contained radioactively labeled protein.

Data analysis. The IC_{50} values for inhibition of enzyme activity or photoaffinity labeling were estimated from least squares best fit polynomial equations fitted to semilogarithmic plots of inhibition data. The NWA Statpak (Northwest Analytical Inc., Portland, OR) was used to perform these calculations.

RESULTS

Initial photolysis experiments. Human kidney TPMT Peak I and Peak II preparations that had been purified by gel filtration chromatography after separation by ion exchange chromatography (see Fig. 1) were exposed to 254 nm UV light in the presence of [^{3}H-*methyl*]Ado-Met. Photoactivation resulted in the radioactive labeling of a protein with a molecular mass of approximately 35 kDa as estimated by SDS–PAGE (Fig. 2). Previous studies of human kidney TPMT had reported the molecular mass of the enzyme to be approximately 36 kDa [2]. Maximum absorbance for Ado-Met occurs at approximately 260 nm. To determine the effect of UV light wavelength on labeling, the initial experiment was repeated using wavelengths of 300 and 350 nm. If the intensity of photoaffinity labeling at 254 nm was arbitrarily defined as 100%, average labeling intensity for the 35 kDa protein at 300 nm was 70 ± 10% (mean ± SD, N = 3), while that obtained at 350 nm was only 1 ± 1%. Therefore, 254 nm was used as the excitation wavelength in all subsequent photoaffinity labeling experiments.

The effect of photolysis time on radioactive labeling of the 35 kDa protein was also studied. The intensity of radioactive labeling of the 35 kDa protein in both Peak I and Peak II preparations increased with increasing time of photolysis for at least 30 min (Fig. 3A). In a parallel experiment, the effect of time of exposure to UV light in the absence of Ado-Met on TPMT enzymatic activity was determined (Fig. 3B). A photolysis time of 30 min was selected for use in subsequent studies since this time offered an acceptable compromise between maximizing

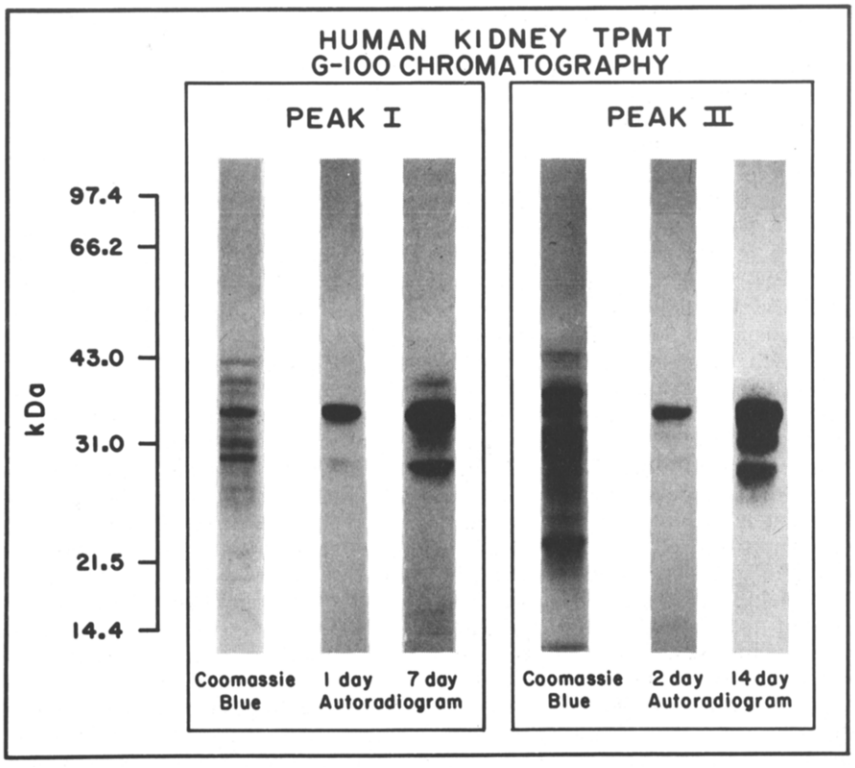

Fig. 2. Photoaffinity labeling with [^{3}H-*methyl*]Ado-Met of human kidney TPMT Peaks I and II purified through gel filtration chromatography. SDS–PAGE of preparations of each isozyme is shown. The left lane in each panel is stained with Coomassie Blue, and the two right lanes are autoradiograms exposed for different times.

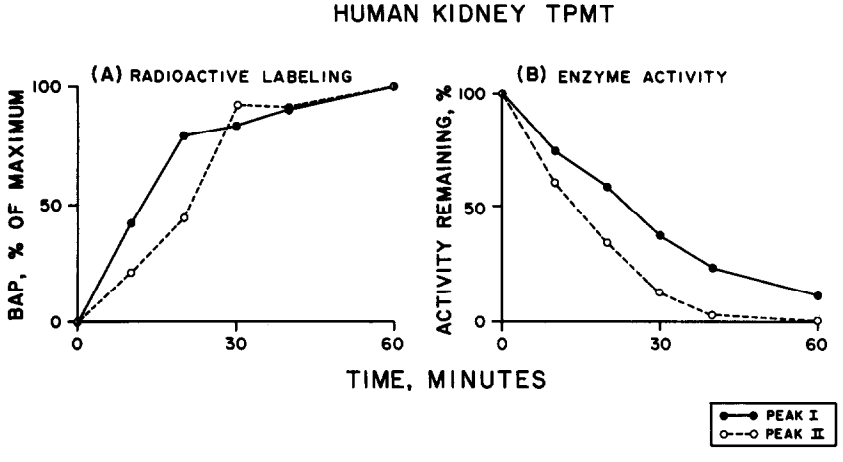

Fig. 3. Effect of photolysis time on human kidney TPMT Peaks I and II. (A) Radioactive labeling of the 35 kDa protein with [^{3}H-*methyl*]Ado-Met, and (B) TPMT enzymatic activity after exposure to UV light in the absence of Ado-Met.

radioactive labeling and minimizing presumed light-induced damage to the protein as estimated by the loss of enzymatic activity (Fig. 3). In summary, these initial experiments had resulted in the establishment of optimal wavelength and photolysis time for the photoaffinity labeling of a 35 kDa protein species in preparations of both isozymes of human kidney TPMT. However, even though the major species labeled in both preparations was a 35 kDa protein, prolonged exposure of the autoradiograms revealed

the existence of additional bands of labeled protein at approximately 40 and 29 kDa for Peak I and at approximately 32 and 29 kDa for the Peak II preparation (Fig. 2). In the case of each isozyme, radioactivity bound to these other species represented less than 10% of the radioactivity associated with the 35 kDa protein.

Photolysis of DEAE fractions. The "minor" radioactive species detected after photoaffinity labeling might have represented other Ado-Met-dependent methyltransferases or Ado-Met "binding proteins" that co-purified with TPMT. That possibility was evaluated by examining the correlation of patterns of radioactive labeling and the elution of TPMT enzymatic activity during the DEAE ion exchange chromatography step that preceded the gel filtration step. The purpose of this experiment was to determine whether TPMT enzymatic activity coeluted with the 35 kDa species. Individual fractions obtained when a pooled 100,000 *g* human renal supernatant was applied to a DEAE ion exchange chromatography column were subjected to photoaffinity labeling. Every fourth fraction in the portion of the NaCl gradient that contained TPMT activity (see Fig. 1) was concentrated with a Centricon-10 microcentrator; aliquots of the concentrated fractions were photolyzed with [^{3}H-*methyl*]Ado-Met; and the photolyzed preparations were subjected to SDS–PAGE. Peaks I and II were separated during ion exchange chromatography (Fig. 4A), but, since only every fourth fraction was studied, separation of the two peaks was not as clearly delineated as is shown in Fig. 1. The elution profile of the photoaffinity labeled 35 kDa protein paralleled that of TPMT enzymatic activity in Peaks I and II (Fig. 4, A and B). However, "overexposure" of the autoradiograms once again demonstrated that proteins with molecular weights other than 35 kDa were photoaffinity labeled, but their elution patterns did not parallel that of TPMT enzymatic activity (Fig. 4C).

Photolysis after hydroxylapatite chromatography. Hydroxylapatite chromatography was then performed after the ion exchange and gel filtration steps to determine whether the 35 kDa protein coeluted with TPMT enzymatic activity during further purification. This step had not been used initially because TPMT enzymatic activity is "lost" when the protein is highly purified [2]. Preparations of Peak I and Peak II isozymes separated by ion exchange chromatography and then purified through gel filtration chromatography were subjected to hydroxylapatite chromatography. Immediately following hydroxylapatite chromatography, photolysis was performed with [^{3}H-*methyl*]Ado-Met. Once again, the predominant radioactively labeled species in both isozyme preparations was a 35 kDa protein (Fig. 5). Other faintly labeled species were still present when the autoradiograms were overexposed (Fig. 5), but these bands were even less prominant than after gel filtration chromatography (Fig. 2). As anticipated, enzymatic activity in the Peak II preparation was very unstable after purification through hydroxylapatite. Only 7% of this enzymatic activity remained after storage for 24 hr at 4°, and only 4% of the activity remained after storage for 24 hr at −80°. Because of rapid loss of TPMT activity after the enzyme was highly purified, all subsequent studies except for the experiment performed with [^{14}C-*carboxyl*]Ado-Met were performed with enzyme purified only through ion exchange and gel filtration chromatography.

[^{14}C-carboxyl]*Ado-Met photoaffinity labeling.* Photoaffinity labeling experiments were also performed using [^{14}C-*carboxyl*]Ado-Met in addition to [^{3}H-*methyl*]Ado-Met with both isozymes of TPMT after purification through hydroxylapatite chromatography. The purpose of these studies was to determine whether radioactive labeling of the 35 kDa protein involved covalent binding of the Ado-Met molecule rather than methylation of the protein by transfer of the ^{3}H-methyl group from [^{3}H-*methyl*]-Ado-Met. Because the specific activity of the ^{14}C-carboxyl compound was only about 0.06% of that of the [^{3}H-*methyl*]Ado-Met, it was necessary to allow these autoradiograms to develop for 12 weeks rather than 1 day. The same 35 kDa protein that was radioactively labeled with [^{3}H-*methyl*]Ado-Met was also radioactively labeled with [^{14}C-*carboxyl*]-Ado-Met for both isozymes (Fig. 6). These results demonstrated that photoaffinity labeling involved the entire Ado-Met molecule rather than methylation of the 35 kDa protein by transfer of the ^{3}H-methyl group. The next series of experiments was performed to determine whether compounds that inhibited TPMT enzymatic activity or served as methyl acceptor substrates for the enzyme were also capable of inhibiting photoaffinity labeling of the 35 kDa protein in both isozymes.

Effects of S-*adenosyl*-L-*homocysteine and sinefungin.* Ado-Hcy is a product of and a competitive inhibitor of all Ado-Met-dependent methyltransferase reactions [22]. The IC_{50} values for the inhibition by Ado-Hcy of the TPMT enzymatic reaction catalyzed by Peak I and II preparations purified through gel filtration chromatography were 3.4 and 2.9 μM, respectively. Concentrations of Ado-Hcy from 1 nM to 1 mM were then tested to determine the possible effect of this compound on photoaffinity labeling. Ado-Hcy inhibited photoaffinity labeling of the 35 kDa protein in each isozyme in a concentration-dependent fashion, with IC_{50} values of 1.6 μM for both Peaks I and II (Fig. 7A). Sinefungin, a structural analog of Ado-Met, is also a potent inhibitor of Ado-Met-dependent methyltransferase enzymes [23]. Sinefungin inhibited TPMT enzymatic activity catalyzed by our preparations with IC_{50} values of 19 and 24 μM for Peaks I and II, respectively. When concentrations from 1 nM to 1 mM were tested, sinefungin also inhibited photoaffinity labeling of the 35 kDa proteins in Peaks I and II in a concentration-dependent fashion, with IC_{50} values of 5.8 and 7.2 μM, respectively (Fig. 7B). It should be pointed out that, because the concentration of Ado-Met used in the photoaffinity labeling experiments was approximately an order of magnitude less than that used to perform the enzymatic assay (see Materials and Methods), IC_{50} values for inhibition of the two processes can only be compared in a qualitative rather than in a quantitative sense.

Effects of 6-*methylmercaptopurine and* 6-*MP.* 6-Methylmercaptopurine, the methylated product of

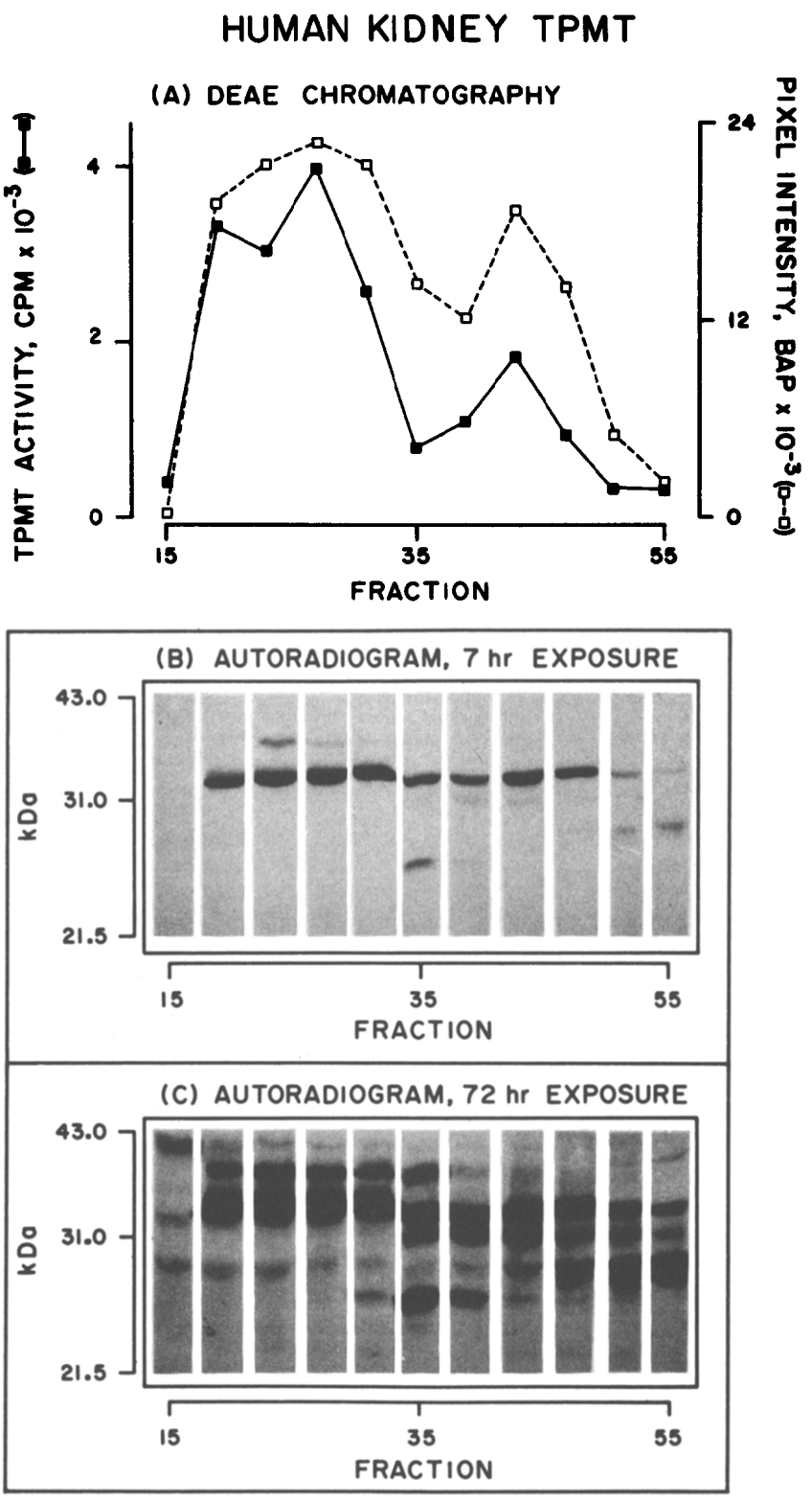

Fig. 4. Photoaffinity labeling with [^{3}H-*methyl*]Ado-Met of DEAE ion exchange chromatography fractions. (A) TPMT enzymatic activity and photoaffinity labeling of the 35 kDa protein in each fraction are shown graphically. (B) and (C) are autoradiograms of the 21.5 to 43 kDa regions of SDS–PAGE lanes for individual ion exchange fractions exposed for 7 (B) and 72 (C) hr, respectively. See text for details.

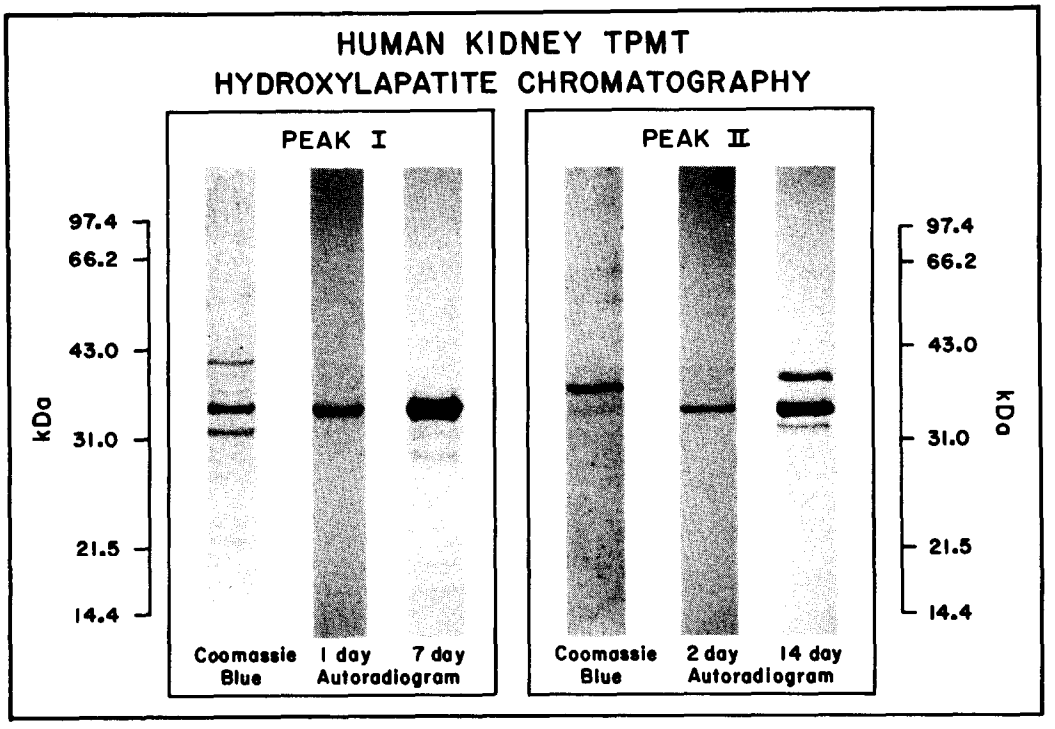

Fig. 5. Photoaffinity labeling with [^{3}H-*methyl*]Ado-Met of human kidney TPMT Peaks I and II purified through ion exchange, gel filtration and hydroxylapatite chromatography. SDS–PAGE of preparations for each isozyme is shown. The left lane in each panel is stained with Coomassie Blue, and the two right lanes are autoradiograms exposed for different times.

the reaction catalyzed by TPMT, is also an inhibitor of the reaction [2]. 6-Methylmercaptopurine inhibited TPMT enzymatic activity for Peak I and II preparations with IC_{50} values of 1.2 and 0.92 mM. respectively. Concentrations of 6-methylmercaptopurine that ranged from 1 μM to 10 mM were tested to determine their effect on photoaffinity labeling of the 35 kDa protein in preparations of each isozyme. 6-Methylmercaptopurine inhibited radioactive labeling of the protein in a concentration-dependent fashion, with IC_{50} values of 3.0 and 2.6 mM for the Peak I and II preparations, respectively (Fig. 8A). The effect on photoaffinity labeling of 6-MP, the methyl acceptor substrate for the reaction, was also tested. Concentrations of 6-MP that ranged from 1 μM to 10 mM were studied, and this compound also inhibited radioactive labeling of the 35 kDa protein in each isozyme in a concentration-dependent fashion, with IC_{50} values of 79 and 35 μM for Peaks I and II, respectively (Fig. 8B).

Effect of DMHBA. DMHBA is a noncompetitive inhibitor of TPMT [2]. DMHBA inhibited TPMT enzymatic activity catalyzed by our Peak I and II preparations with IC_{50} values of 8.2 and 7.3 μM, respectively. When concentrations of DMHBA that ranged from 1 μM to 10 mM were tested to determine whether this compound could also inhibit photoaffinity labeling, it inhibited radioactive labeling of the 35 kDa protein in each isozyme in a concentration-dependent fashion. The IC_{50} values of 240 and 420 μM for inhibition of photoaffinity labeling of Peaks I and II, respectively, were much higher than those required to inhibit enzymatic activity (Fig. 9). These results when combined with those obtained with other TPMT inhibitors and the data demonstrating co-purification of TPMT enzyme activity with the 35 kDa protein in both Peak I and Peak II preparations were compatible with the conclusion that this protein was TPMT.

DISCUSSION

Methylation is an important pathway in the biotransformation of many drugs, xenobiotic compounds and neurotransmitters [24]. Ado-Met is the methyl donor for most of these reactions, including that catalyzed by TPMT. TPMT catalyzes the *S*-methylation of aromatic and heterocyclic thiols including thiopurine drugs such as 6-MP [2, 3, 25]; the level of TPMT activity in human tissue is controlled by monogenic inheritance [4]; and this genetic polymorphism is a major factor responsible for individual variation in the metabolism, toxicity and therapeutic efficacy of thiopurine drugs [8–10]. Efforts to determine the molecular basis for the TPMT genetic polymorphism have been hampered by the loss of TPMT enzymatic activity during purification [2]. Our experiments were performed to determine whether Ado-Met could be used as a photoaffinity ligand for TPMT—and thus make it possible to label the protein radioactively as a first

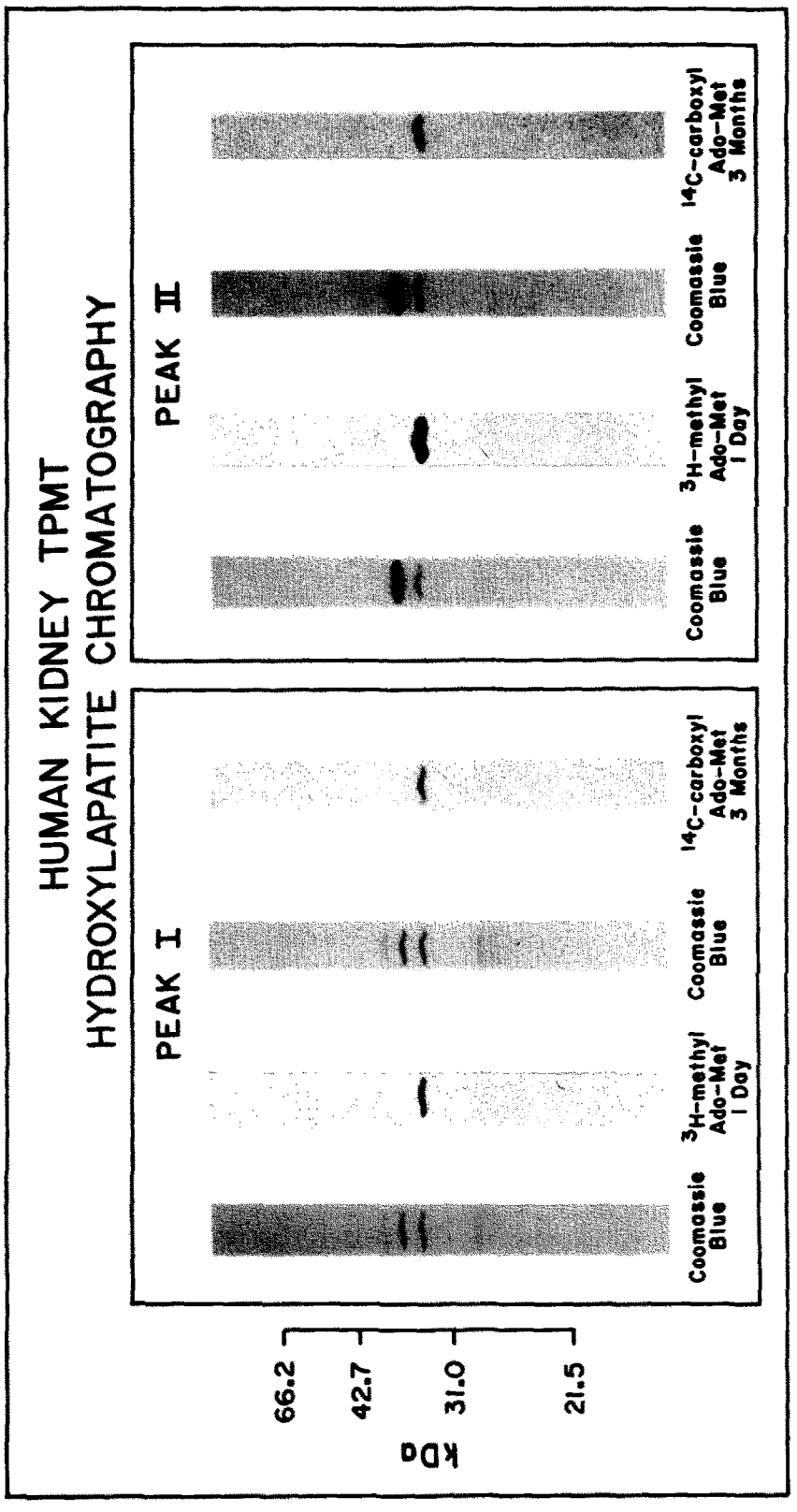

Fig. 6. Photoaffinity labeling with either [^{14}C-*carboxyl*]Ado-Met or [^{3}H-*methyl*]Ado-Met of human kidney TPMT Peaks I and II purified through ion exchange, gel filtration and hydroxylapatite chromatography. *The first and third lanes in each panel are stained with* Coomassie Blue, the second lane is an autoradiogram after photoaffinity labeling with [^{3}H-*methyl*]Ado-Met, and the fourth lane is an autoradiogram after photoaffinity labeling with [^{14}C-*carboxyl*]Ado-Met.

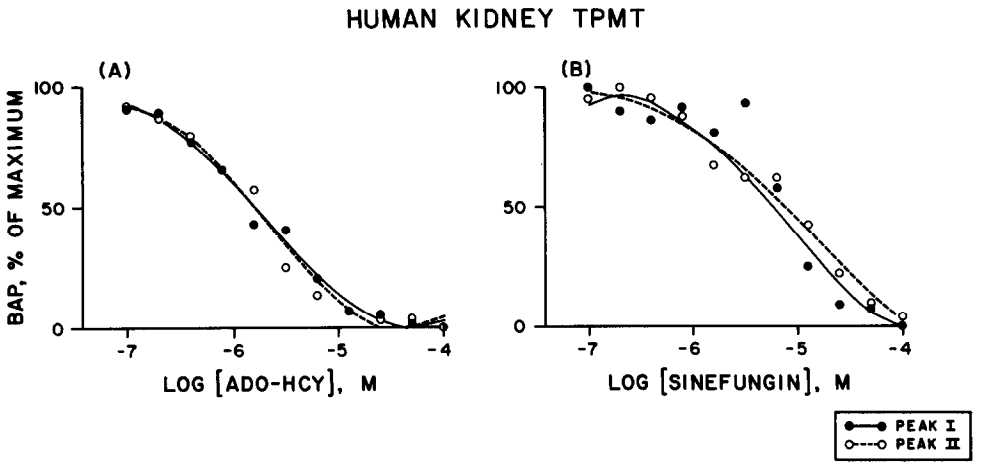

Fig. 7. Effect of *S*-adenosyl-L-homocysteine (Ado-Hcy) and sinefungin on photoaffinity labeling of the 35 kDa protein in each isozyme with [^{3}H-*methyl*]Ado-Met. (A) Effect of increasing concentrations of Ado-Hcy on radioactive labeling of the 35 kDa protein in each isozyme. (B) Effect of increasing concentrations of sinefungin on radioactive labeling of the 35 kDa protein in each isozyme.

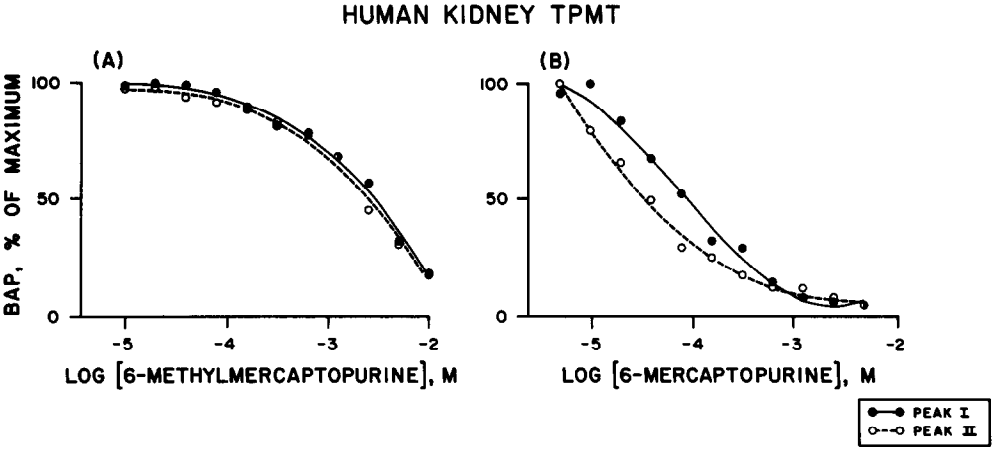

Fig. 8. Effect of 6-methylmercaptopurine and 6-mercaptopurine on photoaffinity labeling of the 35 kDa protein in each isozyme with [^{3}H-*methyl*]Ado-Met. (A) Effect of increasing concentrations of 6-methylmercaptopurine on radioactive labeling of the 35 kDa protein in each isozyme. (B) Effect of increasing concentrations of 6-mercaptopurine on radioactive labeling of the 35 kDa protein in each isozyme.

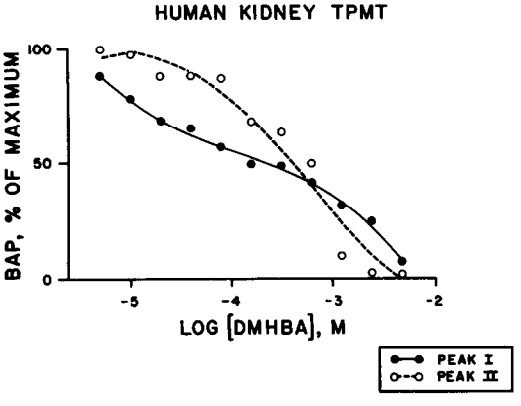

Fig. 9. Effect of 3,4-dimethoxy-5-hydroxybenzoic acid (DMHBA) on photoaffinity labeling of the 35 kDa protein in each isozyme with [^{3}H-*methyl*]Ado-Met. The effect of increasing concentrations of DMHBA on radioactive labeling of the 35 kDa protein in each isozyme is shown.

step toward purification to homogeneity and amino acid sequencing. [^{3}H]Ado-Met has been used as a photoaffinity ligand for other methyltransferase enzymes [15, 16]. It has been speculated that photoactivation of Ado-Met might involve free radical formation at carbon 8 of adenosine—a process known to occur with many purine compounds [15, 16, 26]. The possibility also exists, although it is less likely, that Ado-Met is not itself photoactivated but rather that it might react with a photoactivated amino acid residue at the active site of the enzyme. When Ado-Met was used as a photoaffinity ligand for other enzymes [15, 16], the procedure utilized was complex—irradiation of the enzyme in droplets on a cold glass plate, followed by adsorption of proteins to a paper disc, washing with trichloroacetic acid and extraction with Protosol (New England Nuclear). That procedure was also not selective since it detected any radioactivity labeled protein. Our approach, irradiation of enzyme preparations

in 96-well microtiter plates, followed by SDS–PAGE, was both more convenient and more selective.

We found that exposure of partially purified human kidney TPMT Peak I and Peak II to UV irradiation in the presence of [^{3}H-*methyl*]Ado-Met resulted in the radioactive labeling of a 35 kDa protein in both preparations. The molecular mass of human kidney TPMT as estimated by gel filtration chromatography has been reported previously to be 36 kDa [2], and we estimated a value of 35 kDa from our own gel filtration purification experiments. Therefore, TPMT in the human kidney appears to behave as a monomeric enzyme. The 35 kDa proteins that were radioactively labeled in preparations of each isozyme co-eluted with TPMT enzymatic activity during sequential ion exchange, gel filtration and hydroxylapatite chromatography. Furthermore, inhibitors of TPMT enzymatic activity inhibited photoaffinity labeling of this species in a concentration-dependent fashion. Therefore, the results of experiments conducted with enzyme inhibitors as well as the co-purification of TPMT enzymatic activity with the 35 kDa protein indicated that this protein was TPMT.

The use of Ado-Met for the photoaffinity labeling of TPMT should make it possible to purify the enzyme to homogeneity and to determine amino acid sequences at or near its active site. TPMT is only one of a series of methyltransferase enzymes that show "pharmacogenetic" variation [24]. Included among human methylating enzymes regulated by inheritance are catechol *O*-methyltransferase (EC 2.1.1.6) [27], histamine *N*-methyltransferase (EC 2.1.1.8) [28], and the membrane-bound enzyme thiol methyltransferase (EC 2.1.1.9) [29, 30]. The photoaffinity labeling of ion exchange chromatography fractions of human renal preparations shown in Fig. 4 indicates that photoaffinity labeling with [^{3}H-*methyl*]Ado-Met could be used to identify and purify other Ado-Met-dependent methyltransferases or Ado-Met binding proteins. Therefore, photoaffinity labeling with [^{3}H-*methyl*]Ado-Met should help to make it possible to explore the molecular basis for the TPMT genetic polymorphism as well as the molecular basis for the genetic regulation of other methyltransferase enzymes in humans and in other species [31, 32].

Acknowledgements—We thank Luanne Wussow for her assistance with the preparation of this manuscript. This work was supported in part by NIH Grants GM 28157 and GM 35720 and by NIEHS Contracts ES 05304 and ES 55110.

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