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# THIOPURINE METHYLTRANSFERASE: MOUSE KIDNEY AND LIVER ASSAY CONDITIONS, BIOCHEMICAL PROPERTIES AND STRAIN VARIATION*

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**Abstract**—Thiopurine methyltransferase (TPMT) catalyzes the S-methylation of aromatic and heterocyclic thiol compounds including drugs such as 6-mercaptopurine (6-MP) and 6-thioguanine. In humans, the level of TPMT activity is inherited in a monogenic fashion. It would be important to develop an experimental animal model in which the genetic regulation of TPMT could be studied. Therefore, TPMT activity was measured in kidney and liver homogenates from A/J inbred mice. Apparent Michaelis ($K_m$) constants for the two cosubstrates for the reaction, 6-MP and *S*-adenosyl-L-methionine (Ado-Met), in mouse kidney were $7.0 \times 10^{-4}$ M and $2.4 \times 10^{-6}$ M respectively. Apparent $K_m$ constants for 6-MP and Ado-Met in mouse liver were $5.4 \times 10^{-4}$ M and $2.1 \times 10^{-6}$ M respectively. The pH optimum for the reaction was 6.7 in both tissues, and over 95% of the TPMT activity in both mouse liver and kidney was "soluble" after centrifugation at 100,000 *g* for 1 hr. 3,4-Dimethoxy-5-hydroxybenzoic acid, an inhibitor of human kidney TPMT, decreased mouse kidney and liver enzyme activities by more than 95% at a concentration of 1 mM. TPMT activities were then measured in liver and kidney tissue from nine additional inbred strains of mice aged 7–8 weeks. Six of the nine inbred strains had TPMT activities very similar to those found in A/J animals. However, three strains, the C57BL/6J, C57BL/6ByJ and AKR/J, had significantly lower levels of activity in both liver and kidney than did any of the seven other strains. Liver TPMT activities in these three strains were only 23–32% of the average activity in A/J mouse liver. Kidney enzyme activities in the same three strains averaged 49–62% of the average activity in A/J mouse kidneys. These striking differences in TPMT activity among inbred mouse strains will make it possible to test the hypothesis that inheritance regulates variations in TPMT activity in this experimental animal.

Thiopurine methyltransferase (EC 2.1.1.67, TPMT) catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine, 6-thioguanine and azathioprine [1]. Thiol methylation is an important pathway in the catabolism of these drugs [2], and *S*-methylthiopurine ribonucleotides are "active" metabolites capable of inhibiting purine biosynthesis [3]. A common genetic polymorphism is responsible for wide individual variations in levels of human erythrocyte (RBC) TPMT activity [4]. Human RBC TPMT activity is correlated with the relative level of the enzyme activity in the lymphocyte, platelet and kidney [5, 6]. Therefore, the genetic polymorphism that regulates TPMT activity in the RBC also seems to regulate the enzyme activity in other human cells and tissues. This "pharmacogenetic" polymorphism may be one factor responsible for wide individual variations in the catabolism of thiopurine drugs and in the therapeutic and/or toxic effects of these drugs [7, 8]. It would be very useful if an animal model could be developed in which biochemical mechanisms responsible for the genetic regulation of variations in TPMT activity could be studied. These animals might also be used in pharmacologic and toxicologic experiments.

The present study had two objectives. The first was to determine whether TPMT activity was present in the mouse liver and kidney, and, if so, to study its biochemical characteristics. The second objective was to determine whether large differences in TPMT activity exist among inbred strains of mice. If so, it could be determined whether these differences, like those in humans, are inherited. Inbred mice are an ideal laboratory animal for use in biochemical genetic experiments since there are many well-defined inbred strains and because many recombinant inbred strains of mice are available for genetic linkage studies.

We have found that TPMT activity is present in mouse liver and kidney, and we have determined optimal conditions for the measurement of the enzyme activity in homogenates of both organs. The biochemical characteristics of mouse liver and kidney TPMT are similar to those reported previously for rat and human TPMT [9, 10]. Finally, there are striking variations in TPMT activity among inbred strains of mice.

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## MATERIALS AND METHODS

*Animals.* All mice were purchased from Jackson Laboratory, Bar Harbor, ME. Male A/J mice were used in the initial experiments designed to develop a procedure for the measurement of mouse TPMT and to study the biochemical characteristics of the enzyme in mouse kidney and liver. The animals used in these experiments were 7–10 weeks of age. Ten inbred strains, the A/J, Balb/cByJ, DBA/2J, C3H/HeJ, SWR/J, AKR/J, C57BL/6J, C57BL/6ByJ, C57BR/cdJ, and C57L/J, were then used to study TPMT activities in different mouse strains. Seven- to eight-week-old male and female animals were used in these experiments.

*Tissue preparation.* The mice were fasted for 24 hr with free access to water. They were then killed by cervical dislocation followed by decapitation. Kidneys and livers were removed and placed in ice-cold saline. All subsequent steps were performed at 4°. The organs were weighed and homogenized for 15 sec in 9 vol. (w/v) of 1.15% KCl and 5 mM Tris–HCl, pH 7.8, with a Polytron homogenizer. The homogenates were centrifuged at 15,000 *g* for 15 min, and the supernatant fractions from this step were centrifuged at 100,000 *g* for 60 min. The supernatant fractions after centrifugation at 100,000 *g* were diluted 1:8 (v/v) with 5 mM potassium phosphate buffer, pH 7.5, that contained 0.25% bovine serum albumin. A suspension of Chelex-100 was prepared as described elsewhere [11], and 1 vol. of this suspension was added to 9 vol. of the diluted supernatant fraction from the 100,000 *g* centrifugation step. The suspension was mixed gently by rotation at 12 rpm for 1 hr, and the Chelex-100 was removed by centrifugation at 3300 *g* for 10 min. As discussed previously [12], the chelation step was included to remove magnesium required for the activity of the enzyme hypoxanthine guanine phosphoribosyltransferase. The samples were then diluted for a final time with 5 mM potassium phosphate buffer, pH 7.5, that contained 0.25% bovine serum albumin. The dilution was 1:1 (v/v) for kidney and 1:7 for liver. Therefore, the final dilutions were 200-fold for kidney and 800-fold for liver. Fresh homogenates were used in all of the experiments designed to develop the assay procedure. Studies of TPMT activities in different mouse strains were performed with final, diluted tissue preparations that had been frozen at −80° for 1 day. No change in the enzyme activity occurred under these conditions of storage.

*TPMT assay.* TPMT activity was measured by a modification of the procedure of Weinshilboum *et al.* [12]. 6-Mercaptopurine (6-MP) was used as a substrate for the reaction, and [$^{14}$C-*methyl*]-*S*-adenosyl-L-methionine ([$^{14}$C]Ado-Met) was used as the methyl donor. 6-MP was converted to radioactively labeled 6-methylthiopurine by TPMT. The reaction product was then isolated by organic solvent extraction, and its radioactivity was measured in a liquid scintillation counter.

Specifically, 100-μl aliquots of the final tissue preparations were placed in 15-ml conical stoppered glass centrifuge tubes, and 25 μl of 400 mM potassium phosphate buffer, pH 6.0, was added to each tube. Ten microliters of either dimethyl sulfoxide (DMSO) or DMSO plus 6-MP, 23.6 mg/ml, was added. The samples that contained only DMSO served as blanks. The reaction was initiated by the addition of 25 μl of a mixture of the following reagents (final concentrations in 160 μl indicated): [$^{14}$C]Ado-Met (sp. act. 24 mCi/mmole), $25 \times 10^{-6}$ M; dithiothreitol, $10^{-2}$ M; and allopurinol, $5 \times 10^{-5}$ M. The reaction tubes were incubated in a shaker water bath for 30 min at 37°, and the reaction was terminated by the addition of 0.5 ml of 0.5 M borate buffer, pH 10. Two and one-half milliliters of 20% isoamyl alcohol in toluene was added, and the samples were mixed vigorously on a vortex mixer for 10 sec. After centrifugation at 700 *g* for 10 min in an International model K centrifuge, 1.5 ml of the organic phase was transferred to a 6-ml polyethylene liquid scintillation counting vial that contained 0.5 ml absolute ethanol and 4.0 ml toluene liquid scintillation fluor (5 g of 2,5-diphenyloxazole and 0.1 g 1,4-bis-2-[5-phenyloxazolyl]-benzene per liter toluene). Radioactivity was then measured in a Beckman LS 7500 liquid scintillation counter. All data were corrected for quench and for counting efficiency. The results were also corrected for the extraction of 6-methylthiopurine into the organic phase (41%). One unit of enzyme activity represented the formation of 1 nmole of 6-methylthiopurine/hr. Activity was expressed both as units per g of tissue and as units per mg of protein.

*Protein assay.* Protein concentrations were measured by the method of Lowry *et al.* [13] with bovine serum albumin as a standard.

*Thin-layer chromatography.* The radioactive products of the enzyme reaction were identified by thin-layer chromatography performed with Eastman Chromogram sheets of silica gel [12]. Two different solvent systems were used: 1-butanol, acetone, ammonium hydroxide, and water (50:40:3:15) and 2-propanol, 1-butanol, water and formic acid (60:20:19:1).

*Subcellular distribution studies.* TPMT activity was measured in different fractions of mouse liver and kidney homogenates after differential centrifugation. The homogenates were first centrifuged at 1000 *g* for 10 min. The supernatant fraction that remained after this step was centrifuged at 15,000 *g* for 15 min, and the supernatant fraction was separated from the pellet. The pellet was "washed" twice by resuspension followed by centrifugation. The 15,000 *g* supernatant fraction was centrifuged at 100,000 *g* for 60 min to yield a microsomal fraction. The pellets from each centrifugation step were resuspended in 1.15% KCl that contained 5 mM Tris–HCl, pH 7.8.

Before TPMT activity was measured, 100 μl of each subcellular fraction was mixed with 800 μl of 5 mM potassium phosphate buffer, pH 7.5, that contained 0.25% bovine serum albumin, and 100 μl of a suspension of Chelex-100. These preparations were mixed gently by rotation at 12 rpm for 1 hr at 4°. They were then centrifuged at 3300 *g* for 10 min to remove the Chelex-100. The final supernatant fractions were removed and diluted with the same buffer so that each sample contained approximately the same quantity of tissue protein. Aliquots of 100 μl

were then used to measure TPMT activity. All activities were compared with those present in the original 1000 *g* supernatant fraction.

*Kinetic and statistical analyses.* Michaelis ($K_m$) constants were calculated by the method of Wilkinson [14] with a program written by Cleland [15]. A Hewlett Packard 9845B computer was used to perform these calculations. Differences between means were evaluated by Student's *t*-test for unpaired observations [16].

*Materials.* [$^{14}$C-*Methyl*]Ado-Met (sp. act. 46.0 to 56.2 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and from the Research Products International Corp., Mount Prospect, IL. Ado-Met HCl, tris(hydroxymethyl)aminomethane base, bovine serum albumin, 6-mercaptopurine, 6-methylthiopurine, DMSO, dithiothreitol and allopurinol were purchased from the Sigma Chemical Co., St. Louis, MO. Toluene, isoamyl alcohol, ammonia and 2-propranol were obtained from the J. T. Baker Chemical Co., Phillipsburg, NJ. Formic acid, 1-butanol, Folin reagent and Scintiprep were purchased from Fisher Scientific, Pittsburgh, PA. Chelex-100 was obtained from BioRad Laboratories, Richmond, CA. *p*-Anisic acid and 3,4-dimethoxy-5-hydroxybenzoic acid were purchased from the Aldrich Chemical Co., Milwaukee, WI. Tropolone was obtained from the Regis Co., Morton Grove, IL. SKF-525A was donated by Dr. R. Van Dyke of the Mayo Foundation, Rochester, MN.

## RESULTS

This study consisted of two series of experiments. The first series was designed to determine the properties of mouse kidney and liver TPMT and optimal conditions for the measurement of the enzyme activity. These studies were performed with tissue from A/J mice. The second series of experiments was designed to determine whether there were strain and/or sex differences in mouse TPMT activity.

*Effects of tissue concentration and incubation time on TPMT activity.* TPMT activity increased in a linear fashion with increasing quantities of tissue preparation for both mouse kidney and liver. The relationship between enzyme activity and quantity of diluted tissue supernatant fraction was linear to the equivalent of at least 1.11 mg and 0.56 mg of tissue per assay for kidney and liver respectively. Kidney TPMT activity was measured with supernatant fraction from 0.56 mg of tissue, and supernatant fraction from 0.14 mg of tissue was used to measure mouse liver TPMT activity.

TPMT activity in both tissues also increased in a linear fashion with increasing incubation time for up to 40 min. An incubation time of 30 min was used in all subsequent assays.

*Effect of substrate on TPMT activity.* 6-MP and Ado-Met are the two co-substrates for the TPMT reaction. The effects on enzyme activity of ten concentrations of 6-MP from 0.02 to 10 mM and of nine concentrations of Ado-Met from 0.10 to 25 $\mu$M were determined. Double-reciprocal plots were constructed with these data (Fig. 1, A and B), and apparent $K_m$ constants were calculated. 6-MP concentrations ranging from 0.31 to 10 mM and Ado-Met concentrations ranging from 0.39 to 25 $\mu$M were used to construct the double inverse plots. Apparent $K_m$ constants for 6-MP were $7.0 \times 10^{-4}$ M and $5.4 \times 10^{-4}$ M with kidney and liver respectively. $K_m$ constants for Ado-Met were $2.4 \times 10^{-6}$ M and $2.1 \times 10^{-6}$ M with kidney and liver respectively. The concentrations of both substrates that were used in the assay were at least ten times the calculated apparent $K_m$ constants.

*Effect of pH on TPMT activity.* TPMT activity was measured at several different pH values in kidney and liver preparations from A/J mice (Fig. 2). Potassium phosphate at a final concentration of 62.5 mM was the buffer used. pH values were measured at 20° in the presence of the tissue preparation and all components of the reaction mixture. A bimodal pH curve was found with both tissues (Fig. 2). This

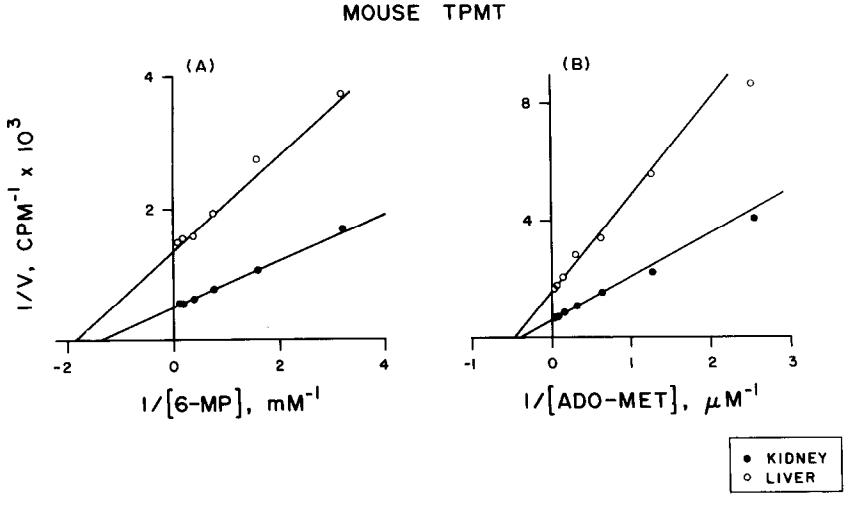


Fig. 1. Double inverse plots of the effects of 6-mercaptopurine (6-MP) and *S*-adenosyl-L-methionine (Ado-Met) concentrations on mouse TPMT. Each point is the mean of three determinations.

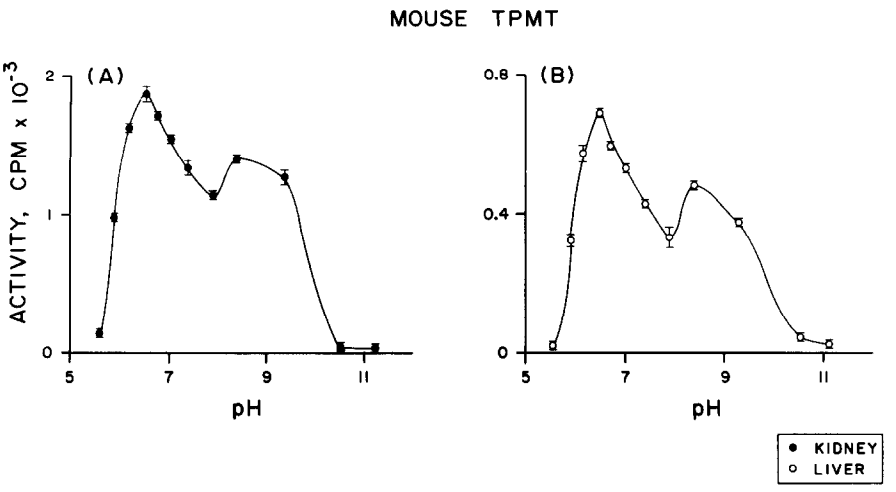


Fig. 2. Effect of pH on mouse kidney (A) and liver (B) TPMT activity. Each point is the mean ± S.E.M. of three determinations.

phenomenon had not been observed in human or rat tissue [9, 10]. With human and rat tissue, a single peak of activity was found at approximately pH 6.7 [9, 10]. In both mouse tissues, the enzyme activity was also maximal at approximately pH 6.7. However, there was a second peak of activity at approximately pH 8.4. These experiments were repeated several times with identical results. Because the major peak of enzyme activity was found at pH 6.7, that pH value was used to perform the routine TPMT assays. The possible meaning of the second peak at pH 8.4 was addressed when the product(s) of the reaction was identified by thin-layer chromatography.

*Thin-layer chromatography of reaction product.* The product of the enzyme reaction was identified by thin-layer chromatography. Organic solvent extracts of the reaction were dried under a stream of nitrogen. This material was applied to silica gel thin-layer sheets prior to separation by thin-layer chromatography [12]. When the reaction was performed at pH 6.7, only a single peak of radioactivity was found for both organs. The $R_f$ value of this radioactive peak was identical to that of authentic 6-methylthiopurine with two different solvent systems: 1-butanol, acetone, $NH_4OH$, and water (50:40:3:15, the "basic" system; Fig. 3, A and B) and 2-propanol, 1-butanol, water and formic acid (60:20:19:1, the "acidic" system). Over 95% of the radioactivity applied to the plates migrated with authentic 6-methylthiopurine in both solvent systems when the assay was performed at pH 6.7.

An attempt was also made to identify the product or products when the reaction was performed at

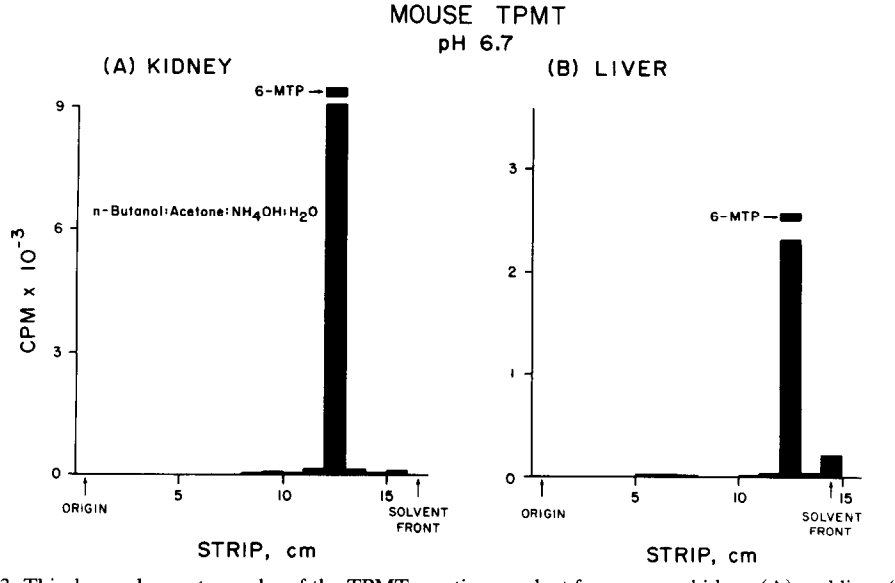


Fig. 3. Thin-layer chromatography of the TPMT reaction product from mouse kidney (A) and liver (B) measured at pH 6.7. The $R_f$ value for 6-methylthiopurine (6-MTP) is shown.

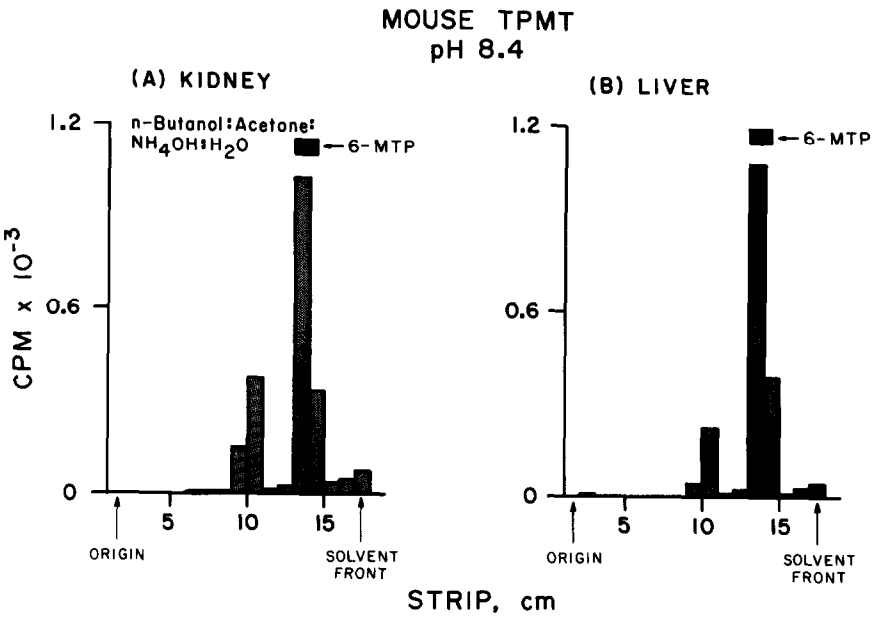


Fig. 4. Thin-layer chromatography of the TPMT reaction product from mouse kidney (A) and liver (B) measured at pH 8.4. The $R_f$ value for 6-methylthiopurine (6-MTP) is shown.

pH 8.4, the pH value at which the "second of the pH maxima" of activity appeared (Fig. 2). When the enzyme reaction was performed at pH 8.4, a radioactive product in addition to 6-methylthiopurine was also present (Fig. 4, A and B). This product migrated more slowly than 6-methylthiopurine in both solvent systems. However, it did not co-migrate with 6-methylthiopurine ribonucleoside, one possible radioactive reaction product that might be formed by the enzyme nucleoside phosphorylase [1]. When kidney preparations were assayed at pH 8.4, the "second" peak accounted for 25 and 38% of the radioactivity applied to the plate in the basic and acidic solvent systems respectively. With liver preparations it was quantitatively smaller, and made up 14 and 6% of the total radioactivity with the two solvent systems respectively. The results of this experiment were compatible with the conclusion that, although 6-methylthiopurine remains the primary reaction product at pH 8.4, an additional methylated metabolite is formed at that pH value. These results also confirmed the wisdom of the choice of pH 6.7, a value at which only 6-methylthiopurine is formed, for the performance of the routine assays. That pH is also the optimal value for tissues from other species [9, 10].

*Subcellular distribution of TPMT activity.* TPMT is a "soluble" activity in human and rat tissues [9, 10]. Therefore, experiments were performed to determine the subcellular localization of TPMT in homogenates of mouse liver and kidney. When differential centrifugation was performed as described in Materials and Methods, TPMT activity was present entirely in the 100,000 *g* supernatant fraction of mouse liver, and 95% of the activity was present in the 100,000 *g* supernatant fraction of kidney preparations.

*Effect of cations and inhibitors on TPMT activity.* Liver and kidney contain several methyltransferase activities including catechol-*O*-methyltransferase (EC 2.1.1.6, COMT) and thiol methyltransferase (EC 2.1.1.9, TMT). COMT is magnesium dependent and is inhibited by calcium [17, 18], while TMT is inhibited by SKF-525A [19]. TPMT activity in human tissue is inhibited by benzoic acid derivatives such as 3,4-dimethoxy-5-hydroxybenzoic acid (DMHBA) and *p*-anisic acid [20]. All three enzymes, COMT, TMT and TPMT, are inhibited by tropolone [10, 19, 21]. A series of experiments was performed to compare the effects of calcium, magnesium and known methyltransferase inhibitors on mouse liver and kidney TPMT activity (Table 1). Calcium and magnesium at 1 mM concentrations, concentrations that, respectively, inhibit and activate COMT, had very little effect on mouse liver and kidney TPMT activity (Table 1). SKF-525A, at a concentration that inhibits TMT in other tissues, also had little effect on TPMT activity (Table 1). However, tropolone significantly inhibited the mouse liver and kidney activity. Two potent TPMT inhibitors, DMHBA and *p*-anisic acid, dramatically decreased mouse kidney and liver TPMT activities (Table 1).

*TPMT activity in inbred strains of mice.* TPMT activities were measured in kidney and liver preparations from ten inbred strains of mice. Whenever possible both male and female animals were studied to determine whether mice, like rats [22], showed sex-related differences in the enzyme activity. Six of the strains had TPMT activities similar to those found in A/J mouse liver and kidney (Figs 5 and 6). However, three strains, the C57BL/6J, C57BL/6ByJ and AKR/J, had significantly less enzyme activity in both tissues. The differences were more striking in liver than in kidney tissue preparations. Average

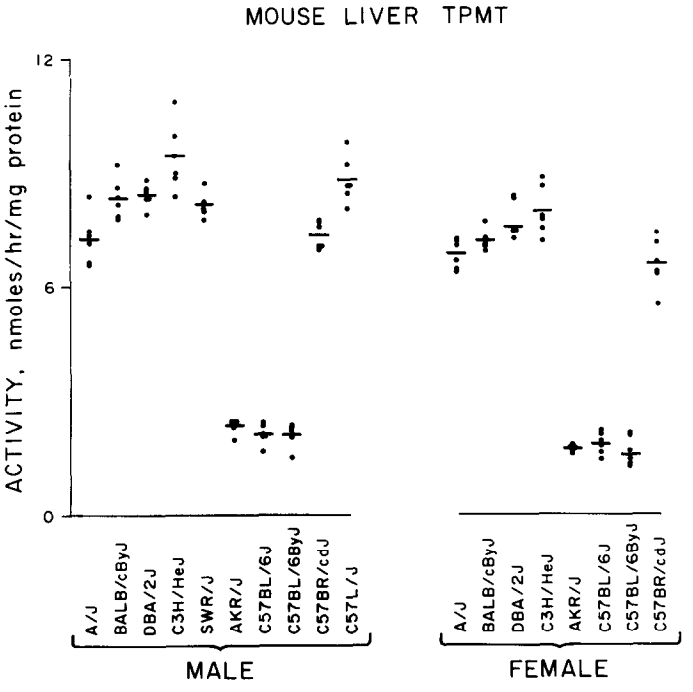


Fig. 5. Liver TPMT activity among inbred strains of mice. Values for male and female animals are shown separately. Each point is from an individual animal and is the mean of three determinations. Bars are mean values for each strain.

liver TPMT activities in these three strains were only 23–32% of the average liver activity in A/J mice (Fig. 5), and average kidney activities in the three strains were 49–62% of the average activity in A/J kidneys (Fig. 6). Relative TPMT activities in these strains were very similar when expressed as either units per g tissue or units per mg protein.

Strain variations in mouse TPMT activity might be due to variations in the presence of endogenous enzyme activators, inhibitors or competing enzyme systems rather than to variations in TPMT activity itself. To test that possibility, two strains with low liver TPMT activities, C57BL/6J and AKR/J, and two strains with high liver TPMT activities, A/J and DBA/2J, were selected for use in "mixing" experiments. Pooled liver supernatant fractions from these strains were mixed in 3 to 1, in 1 to 3, and in equal proportions by volume. TPMT activity was then measured (Table 2). The activities in the mixtures were all quite similar to those anticipated on

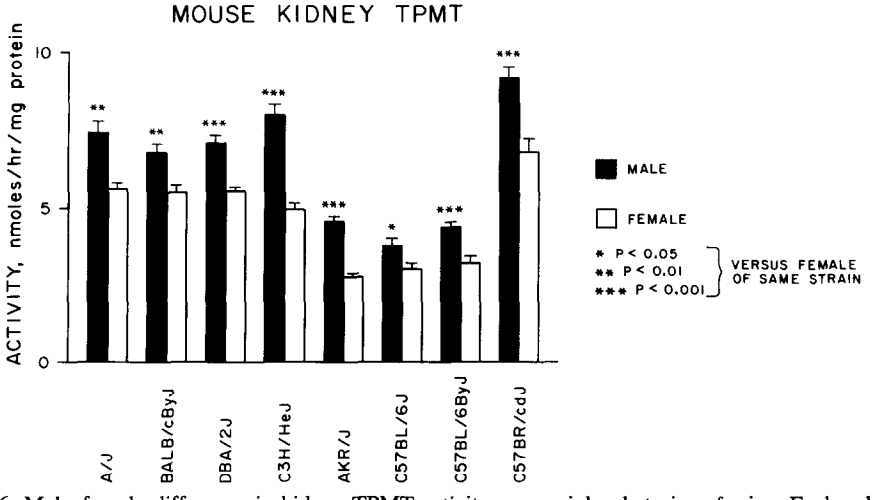


Fig. 6. Male–female difference in kidney TPMT activity among inbred strains of mice. Each value is the mean ± S.E.M. for six mice of each sex. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ males vs females of the same strain.

Table 1. Effects of ions and enzyme inhibitors on mouse TPMT activity*

| Ion or Inhibitor | % Activity remaining | |
|---|---|---|
| | Kidney | Liver |
| $Ca^{2+}$, 1 mM | 106 ± 1 | 105 ± 1 |
| $Mg^{2+}$, 1 mM | 111 ± 1 | 106 ± 2 |
| SKF-525A, 0.05 mM | 104 ± 5 | 103 ± 2 |
| SKF-525A, 0.5 mM | 109 ± 5 | 108 ± 3 |
| Tropolone, 3 mM | 30 ± 1 | 30 ± 1 |
| Tropolone, 30 mM | 13 ± 1 | 11 ± 2 |
| DMHBA, 1 mM | 1 ± 0 | 5 ± 1 |
| *p*-Anisic acid, 1 mM | 8 ± 0 | 8 ± 0 |

* DMHBA is 3,4-dimethoxy-5-hydroxybenzoic acid. $Mg^{2+}$ and $Ca^{2+}$ were used as their chloride salts. Basal activities were 344 and 615 units/g tissue for kidney and liver TPMT respectively. Each value is the mean ± S.E.M. of three determinations.

the basis of simple additive contributions by each of the tissue supernatant fractions. The quantitatively small deviations from this additive relationship could not account for the greater than 3-fold strain differences in enzyme activities. These results make it unlikely that differences in liver TPMT activities among these four mouse strains can be explained on the basis of variations in endogenous enzyme activators, inhibitors, or competing enzyme systems.

*Sex-related differences in mouse TPMT activity.* TPMT activity in the kidneys of adult male Sprague–Dawley rats is at least twice as high as that in the kidneys of female Sprague–Dawley rats [22]. This difference is testosterone dependent [22]. Therefore, we examined the possibility that there might be sex-related differences in TPMT activity in mouse kidney and liver. Data from both sexes were available for only eight of the ten strains that we studied. In all eight strains, TPMT activity in kidney preparations from female animals was significantly less than that present in preparations from male animals (Fig. 6). The differences ranged from 19.2 to 39.8% less renal TPMT activity in female than in male animals. In six of the eight strains, hepatic TPMT activity levels were also significantly ($P < 0.05$) lower in female than in male animals. These differences ranged from 4.6 to 25.5% less TPMT activity in the livers of female animals.

## DISCUSSION

TPMT plays an important role in the catabolism of thiopurine and thiopyrimidine drugs [1, 2, 23]. This enzyme also catalyzes the S-methylation of aromatic sulfhydryl compounds such as thiophenol [20]. S-Methylated thiopurine ribonucleotides are active metabolites that can inhibit purine biosynthesis [3]. TPMT is present in the human RBC [12] where its activity is regulated by a common genetic polymorphism [4]. This pharmacogenetic variation in the RBC enzyme activity reflects variations of TPMT activity in other human tissues and cells [5, 6]. These observations have led to the suggestion that individual variations in TPMT activity may represent one factor responsible for individual differences in the therapeutic effects or toxicity of thiopurine drugs [7, 8]. It is important to develop experimental animal models that can be used to study the genetic regulation of TPMT and to study the pharmacologic implications of variations in this enzyme activity.

The objectives of the present experiments were, first, to determine whether TPMT activity is present in mouse liver and kidney, and, if so, to study the biochemical properties of the enzyme. The second purpose was to determine whether there are significant variations in TPMT activity among inbred strains of mice. TPMT activity was present in A/J mouse liver and kidney, and an assay procedure for the measurement of the enzyme activity in those tissues was developed. $K_m$ constants for the two cosubstrates for the reaction, 6-MP and Ado-Met, as well as the pH optima for the reaction were similar in mouse kidney and liver. In addition, the biochemical characteristics of TPMT in these mouse tissues were similar to those of TPMT in human and rat tissues. For example, the $K_m$ constants in rat and human kidney were 1.4 and 0.30 mM for 6-MP and 4.3 and 2.7 $\mu$M for Ado-Met, respectively [9, 10], values very similar to those found with mouse tissue. The subcellular distribution of mouse TPMT and the effects of inhibitors on the mouse enzyme activity were identical to those of TPMT in other species [9, 10]. However, the bimodal pH curve for mouse

Table 2. Mouse liver TPMT "mixing" experiments*

| Mixture ratio | Percent of "expected" activity | | | |
|---|---|---|---|---|
| | $H_1 + L_1$ | $H_1 + L_2$ | $H_2 + L_1$ | $H_2 + L_2$ |
| High : Low | | | | |
| 3:1 | 110 ± 3 | 101 ± 2 | 100 ± 2 | 107 ± 2 |
| 1:1 | 100 ± 3 | 102 ± 3 | 99 ± 2 | 103 ± 4 |
| 1:3 | 90 ± 4 | 95 ± 4 | 95 ± 4 | 97 ± 2 |

* Volume ratios of 3:1, 1:3, and 1:1 were made with pooled liver supernatant fractions from three individual male animals of strains with high ($H_1$ = A/J, $H_2$ = DBA/2J) and low ($L_1$ = C57BL/6J, $L_2$ = AKR/J) TPMT activities. TPMT activity was measured in each mixture. Each value is the mean ± S.E.M. of three determinations, expressed as a percentage of the value "expected" on the basis of a simple additive contribution by each supernatant fraction. Basal activities were 169, 163, 508 and 550 units/g tissue for the $L_1$, $L_2$, $H_1$ and $H_2$ preparations respectively.

TPMT activity had not been reported with either human or rat tissue [9, 10]. The second of the apparent "pH maxima" at pH 8.4 was due in part to the formation of a methylated metabolite other than 6-methylthiopurine. That metabolite was not identified. However, at pH 6.7, the optimal value for mouse, human and rat tissue, only the expected product of the TPMT reaction was formed. Therefore, pH 6.7 was used in all of our other experiments.

TPMT activity was also measured in livers and kidneys from ten inbred strains of mice. Six strains had activities similar to those found in A/J mice. Three strains, the C57BL/6J, C57BL/6ByJ and AKR/J, had significantly lower levels of activity in both liver and kidney than did any of the other seven strains studied (Figs 5 and 6). Male mice, like male rats [22], had higher levels of renal TPMT activity than did female animals. However, these differences were not as striking as the 2-fold higher levels of activity in the kidneys of male Sprague–Dawley rats compared with the kidneys of female Sprague–Dawley rats [22].

It will now be possible to perform breeding experiments to test the hypothesis that strain differences in mouse TPMT activity are inherited. If they are, as is likely, it should be possible to determine the mode of inheritance. If strain variations in mouse TPMT activity do prove to be genetic, it is of interest that the closely related C57BL/6J and the C57BL/6ByJ strains both have low levels of activity. However, since the C57BR/cdJ and C57L/J strains have high activity, the hypothetical mutation responsible for low activity must have occurred after divergence of the brown from the black C57 subline [24]. The AKR, another "low activity" strain, arose from a completely different line than did the C57BL strains [24]. If breeding experiments do demonstrate inherited strain variations in mouse TPMT activity, it will be possible to study the biochemical basis for that effect. Finally, it is particularly fortunate that the DBA/2J and C57BL/6J strains differ in their enzyme activities, since many recombinant inbred lines are available for these two strains [25]. Recombinant inbred animals could be used for possible genetic linkage studies and for experiments designed to evaluate the consequences of differences in TPMT activity for the toxicity and/or therapeutic effects of thiopurine drugs. In summary, our experiments represent a step toward increasing our understanding of the regulation of an important drug-metabolizing enzyme activity. These results may make it possible to study the biochemical basis for inherited variations in TPMT activity and to study the possible relationship of those variations to individual differences in the response to, and the toxicity of, thiopurine and thiopyrimidine drugs.

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