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SEQUENTIAL METHYLATION OF 2-MERCAPTOETHANOL TO THE DIMETHYL SULFONIUM ION, 2-(DIMETHYLTHIO)ETHANOL, *IN VIVO* AND *IN VITRO*

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Abstract—Thioether methyltransferase (*S*-adenosyl-L-methionine: thioether *S*-methyltransferase; EC 2.1.1.96) catalyzes the methylation of X in compounds of the type R-X-R′(X = S, Se, Te), yielding a methyl onium ion. Previous results using mice have demonstrated a role for thioether methyltransferase in the conversion and clearance of thioethers by methylation to more water-soluble methyl sulfonium ions suitable for excretion in the urine. A potential major physiological source of thioethers is reactions catalyzed by microsomal thiol methyltransferase (*S*-adenosyl-L-methionine: thiol *S*-methyltransferase; EC 2.1.1.9), which has been shown to methylate a diverse range of aliphatic sulfhydryl compounds. This study provides evidence for the sequential methylation of the aliphatic thiol, 2-mercaptoethanol, first to the methyl thioether, 2-(methylthio)ethanol, by thiol methyltransferase followed by methylation of this methyl thioether to the dimethyl sulfonium ion, 2-(dimethylthio)ethanol, by thioether methyltransferase. This sequence of reactions was demonstrated *in vivo* by injecting mice i.p. with radioactive 2-mercaptoethanol and analyzing the labeled methylated products, 2-(methylthio)ethanol and 2(dimethylthio)ethanol, in the urine by HPLC. In addition, the system converting 2-mercaptoethanol to 2-(dimethylthio)ethanol was reconstituted *in vitro* using solubilized mouse liver microsomes as a source of thiol methyltransferase and purified thioether methyltransferase from mouse lung. The results of these *in vivo* and *in vitro* studies established the sequential methylation of 2-mercaptoethanol by these two enzymes.

Key words: sulfur methylation; thiol and thioether methyltransferases; biosynthesis of methyl sulfonium ions; 2-mercaptoethanol metabolism; *S*-adenosylmethionine; sulfur detoxification

AdoMet§-dependent methylation plays a major role in the metabolism and elimination of sulfur compounds in animals. Sulfur methylation is important in the biotransformation of aliphatic and aromatic sulfhydryl drugs such as the antirheumatic medication D-penicillamine [1] and the antineoplastic agent 6-mercaptopurine [2]. This biochemical process can also participate in the metabolism of endogenous sulfhydryl compounds (hydrogen sulfide and methyl mercaptan) as well as the detoxification of xenobiotics [3–5]. One enzyme involved in AdoMet-dependent methyl conjugation to various sulfur-containing compounds is thioether methyltransferase (EC 2.1.1.96), which was discovered during studies of selenium metabolism and detoxification [6]. Thioether methyltransferase, a cytosolic monomeric protein primarily localized in mammalian liver and lung, appears to be the sole enzyme responsible for trimethyl selenonium ion synthesis in mice [6]. Purified thioether methyltransferase also methylates dimethyl telluride, dimethyl sulfide, and various other compounds with thioether bonds [3, 6].

Mice treated with various thioethers were found to excrete the corresponding methyl sulfonium ion in their urine [3]. This supports the hypothesis that the physiological role of thioether methyltransferase is to enhance the clearance of thioethers from the body by methylation to more water-soluble sulfonium ions suitable for urinary excretion. Because the enzyme transfers methyl groups to various thioether substrates *in vitro* and *in vivo*, it is of interest to consider potential sources of such substrates. Thioethers are commonly ingested as environmental contaminants or may be generated physiologically by reduction of sulfoxides. Another potential physiological source of thioethers is reactions catalyzed by thiol *S*-methylating enzymes, which have been shown to methylate a diverse range of exogenous sulfhydryls [7–10]. Thiol methylation in mammals is catalyzed by two separate AdoMet-dependent enzymes, thiopurine methyltransferase (EC 2.1.1.67) and thiol methyltransferase (EC 2.1.1.9). These two enzymes differ in their subcellular locations, substrate specificities, inhibitor sensitivities, and regulation. Thiopurine methyltransferase is a cytoplasmic enzyme that preferentially catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds [10, 11], whereas thiol methyltransferase is membrane-bound with a substrate preference for aliphatic and "non-

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§ Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; 2-ME, 2-mercaptoethanol; MTE, 2-(methylthio)ethanol; DMTE, 2-(dimethylthio)ethanol; Adox, periodate-oxidized adenosine, also known as adenosine-2′,3′-dialdehyde; $C_{12}E_{10}$, polyoxyethylene 10-lauryl ether; PMSF, (phenylmethyl)sulfonyl fluoride; and DEAE, (diethyl)aminoethyl.

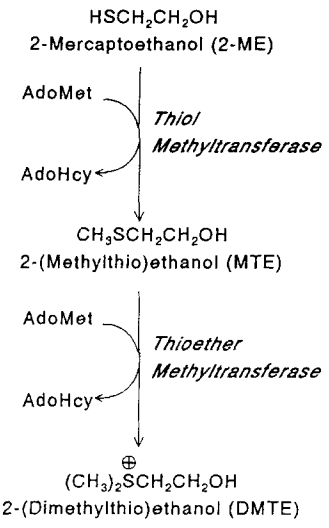

Fig. 1. Sequential methylation pathway converting 2-ME to DMTE. 2-ME is proposed to be methylated by two AdoMet-requiring steps to its final product, DMTE, by the actions of thiol methyltransferase and thioether methyltransferase.

physiologic" sulfhydryls [7, 12, 13]. Aliphatic thiol substrates that undergo methylation by thiol methyltransferase include 2-ME and methyl mercaptan [5, 7]. The respective products of these reactions, MTE and dimethyl sulfide, are substrates for thioether methyltransferase [6]. Therefore, a primary physiological role of thioether methyltransferase in sulfur metabolism may be to methylate not only exogenous thioethers but also to add a final methyl group to the methyl thioether products generated endogenously by thiol methyltransferase. This hypothesis is summarized for 2-ME in Fig. 1, and its substantiation is presented below.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]AdoMet (11 Ci/mmol) was obtained from New England Nuclear (Boston, MA), [1,2-^{14}C]2-ME (1.4 Ci/mol) from Sigma (St. Louis, MO) and [^{14}C]CH_3I (54 Ci/mol) from Amersham (Arlington Heights, IL). $C_{12}E_{10}$, Triton X-100, AdoMet, AdoHcy, and 2-ME were purchased from Sigma, whereas CH_3I and MTE were obtained from Aldrich Fine Chemicals (Milwaukee, WI). Adox was prepared from adenosine by the method described previously [14]. Female Swiss-Webster albino mice, weighing 22–25 g (Charles River Laboratories) and fed a standard commercial diet, were used for *in vivo* metabolism studies and as a source of thiol methyltransferase (liver) and thioether methyltransferase (lung). HPLC was performed using a Spectra-Physics system consisting of an SP8800 pump, an SP8450 detector, and an SP4290 integrator with an SP8780 autosampler. System control and data capture were provided by Spectra-Physics Chromstation/2 software run with an IBM PS/2 model 50 computer. C_{18} reversed-phase columns (0.46 × 25 cm) and SCX cation-exchange columns (0.46 × 25 cm) were from Supelco. ^{3}H and ^{14}C samples were diluted with Ecolite(+) scintillation fluid (ICN Biomedicals, Costa Mesa, CA) and counted on a Beckman LS-3100 liquid scintillation counter.

Synthesis of [methyl-^{14}C]*DMTE and* [methyl-^{3}H]-*MTE standards.* To prepare [*methyl*-^{14}C]DMTE as a standard for comparison with metabolites during HPLC analysis, [*methyl*-^{14}C]CH_3I (54 Ci/mol) was dissolved in acetic acid at 1 μCi/μL. A 10-μL portion of this solution was added to 10 μL MTE and incubated for 24 hr at room temperature. The solution was dried in a stream of air, the residue was washed twice with diethyl ether, and the remaining residue was dissolved in 5% perchloric acid at 2000–5000 cpm/μL. The structure of the chemically synthesized DMTE was confirmed by ion chromatography and low-resolution fast-atom bombardment mass spectrometry, as previously described [15].

Radiolabeled MTE was enzymatically prepared using thiol methyltransferase with [*methyl*-^{3}H]-AdoMet as the methyl donor and 2-ME as the methyl acceptor. Reaction mixtures were prepared and fractionated by HPLC Method 2 as described below. The eluate between 8.0 and 9.5 min was collected, and perchloric acid was added to a final concentration of 5%. The authenticity of [*methyl*-^{3}H]MTE was confirmed by mixing with unlabeled MTE and finding matching retention times for peaks of ^{3}H and absorbance at 214 nm by both HPLC Methods 1 and 2. The [*methyl*-^{3}H]MTE was used as a standard during HPLC analysis for peak identification of MTE produced *in vitro* and *in vivo*.

In vivo *metabolism of 2-ME.* [1,2-^{14}C]MTE and [1,2-^{14}C]DMTE were measured in the urine of mice treated with [1,2-^{14}C]2-ME in 0.9% NaCl. The solution was injected i.p. (225 μL) at a concentration of 50 mM 2-ME, containing 5 μCi of the radioactive 2-ME (adjusted with unlabeled 2-ME to 0.4 Ci/mol) and yielding doses of 0.2 μCi/g and 450 μmol/kg body weight (mice weighed 25 g each). This dose was chosen to give a concentration of 2-ME in body water of about 600 μM (assuming a mouse to be about 75% water). This would saturate the high-affinity form of thiol methyltransferase and give measurable activity with the low-affinity form, if the kinetics of this enzyme are biphasic *in vivo* as they are *in vitro* [16]. The same volume of 0.9% NaCl was injected in control mice. One additional group of mice was injected with 200 μL of 10 mM Adox in saline (80 μmol Adox/kg body weight) 30 min prior to injection with the radioactive 2-ME solution. This dose of Adox was found to give maximal accumulation of AdoHcy in livers of treated mice [14] and to strongly inhibit selenium methylation [17] and thioether methylation [3] in mice. Three mice were used in each treatment group. After the injections, each mouse was placed in a glass metabolism cage, and urine was collected for 24 hr on ice (to prevent bacterial growth). The urine samples were brought to 5% perchloric acid and placed on ice for 2 hr. Samples were deproteinized by centrifugation at 10,000 *g* for 3 min. Samples of 50 μL of supernatants

were analyzed using HPLC Method 1 as described below.

HPLC analyses of sequential methylation products (Method 1). A C_{18} reversed-phase column was used to separate radioactive 2-ME, MTE, and DMTE (retention times: 3, 4.8, and 10.5 min, respectively). The column was eluted at room temperature at a flow rate of 2 mL/min, starting with 10 mM octyl sulfonate in 20 mM ammonium phosphate (pH 6.0) at time zero followed by a gradient of 1% acetonitrile/min for 15 min (HPLC Method 1). After completion of a run, the system was re-equilibrated for 7 min with the starting buffer before injection of the next sample. Addition of the ion-pairing agent, octyl sulfonate, was necessary to obtain retention of the DMTE sulfonium ion. The column eluate was monitored at 214 nm, and 1-mL fractions were collected every 0.5 min, diluted with 8 mL of scintillation fluid, and counted for radioactivity.

Recoveries and retention times were assessed by spiking nonradioactive urine samples with known amounts of chemically synthesized [*methyl*-^{14}C]-DMTE and enzymatically prepared [*methyl*-^{3}H]-MTE. Commercially obtained nonradioactive standards of 2-ME and MTE were also added and could be detected by UV monitoring at 214 nm [18]. Recoveries of the two methylated metabolites were always greater than 95%. When radioactive urine samples were chromatographed, 90–98% of the total radioactivity applied to the column was recovered.

HPLC analysis of AdoMet and AdoHcy. AdoHcy and AdoMet were measured in deproteinized supernatants of the perchloric acid-treated urine samples. Samples of 50 μL of the acid-soluble supernatants were fractionated by cation-exchange HPLC on a 0.46 × 25 cm Supelco SCX column. The column was eluted at 2 mL/min by a 10-min linear gradient from 10 mM ammonium formate (pH 3.0) in 20% acetonitrile to the same buffer plus 50 mM ammonium sulfate. Column elution continued isocratically for an additional 5 min with the latter buffer. The eluate was monitored at 258 nm, and the AdoHcy and AdoMet, eluting at 7.5 and 12.6 min, respectively, were quantified by electronic integration of peak areas using response factors determined with authentic standards run under the same conditions as the biological samples.

Thiol methyltransferase preparation and solubilization. Mice were killed by cervical dislocation, and their livers were excised and placed in ice-cold saline. All subsequent steps were performed at 4°. Livers were weighed and homogenized in 9 mL/g cold buffer A (250 mM sucrose, 50 mM HEPES, 5 mM Tris–HCl buffer, 1 mM EDTA, 0.15 M KCl, and 5% glycerol, pH 7.8) using a Polytron homogenizer. The homogenate was centrifuged at 15,000 *g* for 12 min followed by centrifugation of the acquired supernatant fraction at 100,000 *g* for 60 min. The cytosolic supernatant was removed and used for thioether methyltransferase assays (described below), and the microsomal pellet was resuspended at a protein concentration of approximately 5 mg/mL in buffer A containing 0.1 mM PMSF, 20% glycerol, and 0.35% $C_{12}E_{10}$. After 30 min this mixture was centrifuged at 100,000 for 45 min, and the supernatant was retained as a source of thiol methyltransferase. In preliminary experiments, this concentration of detergent was found to give optimal solubilization and stabilization of thiol methyltransferase.

Measurement of thiol methyltransferase activity was performed by a modification of previously described techniques by monitoring the conversion of 2-ME to MTE [16, 18]. The assay, based on the first reaction of the sequence illustrated in Fig. 1, follows the transfer of radioactivity from 0.1 mM [*methyl*-^{3}H]AdoMet (500 Ci/mol, 0.05 mCi/mL) to 1 mM 2-ME upon incubation with thiol methyltransferase (10–250 μg protein) in a reaction volume of 100 μL containing 50 mM HEPES, 5 mM Tris–HCl (pH 7.8), 1 mM EDTA, and 20% glycerol. Reactions were stopped after 15 min at 37° by the addition of 20 μL of 30% perchloric acid and deproteinized by centrifugation at 10,000 *g* for 3 min. Samples (50 μL) of the acid-soluble supernatants were analyzed by using a C_{18} reversed-phase column, eluted at 2 mL/min with deionized water (HPLC Method 2). This separated the product [*methyl*-^{3}H]-MTE, eluting at 8.7 min, from unreacted [*methyl*-^{3}H]AdoMet, eluting near the void volume. During routine analyses, only the product [*methyl*-^{3}H]MTE was collected between 8 and 10 min and counted. Three different types of blank samples were used in determining true thiol methyltransferase activity [18, 19]: (i) a "no methyl acceptor substrate–no enzyme" blank that accounted for radioactive contaminants in the methyl donor, [*methyl*-^{3}H]-AdoMet, and typically gave less than 90 cpm; (ii) a "no methyl acceptor substrate" blank that measured nonspecific methyltransferase activity and typically gave about 4000 cpm; and (iii) a "no enzyme" blank that tested for the nonenzymatic interaction between AdoMet and 2-ME under these conditions and typically gave less than 1000 cpm. Thiol methyltransferase activity was corrected by subtracting the sum of the blanks from total activity values [19]. Combined blank values were less than 10% of uncorrected total thiol methyltransferase activity, which was usually greater than 100,000 cpm. One unit of enzyme activity represented the formation of 1 nmol MTE/min of incubation at 37°. Specific activity was expressed as units per milligram of protein.

Assay for thioether methyltransferase. Thioether methyltransferase activity was measured in crude homogenates and 15,000 *g* supernatant fractions similar to the method described previously [6] and based on the second reaction of the sequence in Fig. 1. Reaction mixtures of 100 μL contained 1 mM MTE, 0.1 mM [*methyl*-^{3}H]AdoMet (500 Ci/mol, 0.05 mCi/mL), 1 mM EDTA, and 25 mM HEPES (pH 6.3). Blanks contained all components except MTE. Reaction mixtures were incubated for 15 min at 37° and were stopped by the addition of 20 μL of 30% perchloric acid. Following centrifugation at 10,000 *g* for 3 min, samples (50 μL) of the acid-soluble supernatants were analyzed on a C_{18} reversed-phase column by HPLC Method 1 (described above). The eluate was monitored at 258 nm, 1-mL fractions were collected and diluted with 8 mL of scintillation fluid, and the [*methyl*-^{3}H]DMTE was determined by liquid scintillation counting. One unit of enzyme

activity was expressed as the formation of 1 nmol of DMTE/min of incubation at 37°.

Purification of thioether methyltransferase. Purification of thioether methyltransferase from mouse lung to homogeneity was accomplished by sequential chromatography on DEAE-Sephacryl ion exchange, Sephadex G-75 gel filtration, and chromatofocusing (pH 4–7) [6]. This procedure typically gives a 650-fold purification yielding 1 mg of pure enzyme from 40 pairs of lungs (*ca.* 20 g wet tissue).

Sequential methylation in vitro *of 2-ME.* The sequential methylation of 2-ME by the enzymes thiol methyltransferase and thioether methyltransferase to the methyl thioether MTE and ultimately the dimethyl sulfonium ion DMTE is based on the sequence of reactions in Fig. 1. Reaction mixtures of 100 μL contained $C_{12}E_{10}$-solubilized microsomal thiol methyltransferase (4.44 mg/mL, 0.75 U activity) and purified thioether methyltransferase (2.44 mg/mL, 0.25 U activity) in 50 mM HEPES (pH 7.2), 1 mM EDTA, 0.05 mM $C_{12}E_{10}$, and either 1 mM [*methyl*-^{3}H]AdoMet (500 Ci/mol) and 4 mM 2-ME (Experiment A), or 4 mM [1,2-^{14}C]2-ME (0.4 Ci/mol) and 1 mM nonradioactive AdoMet (Experiment B). After 30 min at 37° reactions were stopped with 20 μL of 30% perchloric acid, and the protein precipitate was removed by centrifugation at 10,000 *g* for 3 min. Samples (50 μL) of the acid-soluble supernatants were analyzed by HPLC Method 1. Fractions were collected every 0.5 min and counted for the respective radiolabel. To determine if both methyltransferases were necessary for complete methylation, controls included reactions with neither enzyme, thiol methyltransferase only, and thioether methyltransferase only. Experiments were performed in triplicate, and HPLC profiles from a representative experiment are presented in Fig. 3 with a quantitative summary shown in Fig. 4.

The choice of conditions for the sequential methylation reactions was based on the following considerations. The goal of the present study was to demonstrate the sequential methylation of a thiol to its respective dimethyl sulfonium ion using a system that most closely mimics the metabolic pathway *in vivo*. In every mammalian species studied, hepatic microsomes contain two forms of thiol methyltransferase activity differing in their substrate kinetics and pH optima [16, 18–20]. The high-affinity enzyme has a K_m for 2-ME of 9–30 μM and an optimal pH range from 7.2 to 8.1, whereas the low-affinity thiol methyltransferase has a K_m for 2-ME of at least 20 mM and acts optimally within a pH range from 9.0 to 9.5. This latter pH range is not physiological and would promote the degradation of AdoMet. Moreover, the high concentrations of substrate needed to see half-maximal activity of low-affinity thiol methyltransferase may introduce error into the *in vitro* methylation system because of nonspecific methylation. Thioether methyltransferase has a pH optimum of 6.3 and retains considerable activity at pH 7.2, but activity is reduced above pH 7.8 [6]. Therefore, the sequential methylation reactions were buffered at the near-physiological pH of 7.2, which represents a compromise between optimal pH values of the two methyltransferases. The 3:1 ratio of thiol methyltransferase to thioether methyltransferase in these reactions was equal to that measured in a 15,000 *g* supernatant preparation from crude mouse liver homogenate.

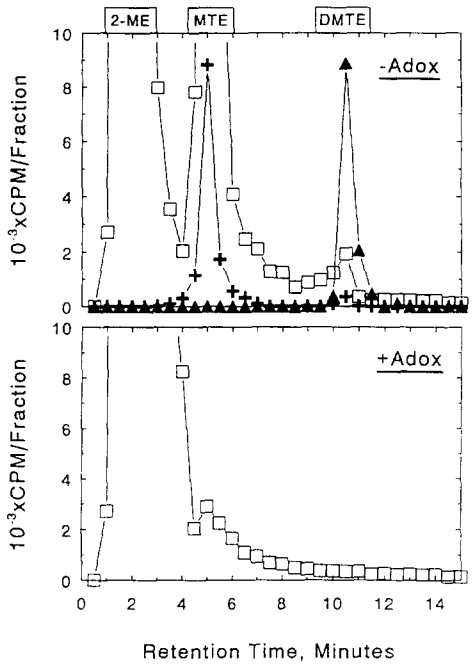

Fig. 2. HPLC elution profiles of ^{14}C-containing metabolites in urine from mice injected with [1,2-^{14}C]2-ME. Mice previously untreated (top panel) or pretreated with Adox (bottom panel) were injected with [1,2-^{14}C]2-ME, and their urine samples were collected and fractionated by HPLC Method 1 as described in Materials and Methods. Symbols: urine samples (□); [*methyl*-^{3}H]MTE standard (+); and [*methyl*-^{14}C]DMTE standard (▲).

Thiol methyltransferase activity was linear with respect to enzyme protein (10–250 μg protein) and time of incubation (55 min). To ensure that there was a linear relationship between activity and time of incubation, all assays were performed at 30 min.

Protein determination. The amount of protein was measured by the method of Spector [21]. Bovine serum albumin was used as a standard.

RESULTS

Sequential methylation in vivo *of 2-ME to MTE and DMTE.* Representative HPLC elution profiles of urine samples collected from mice treated with [1,2-^{14}C]2-ME are shown in Fig. 2. The HPLC elution profile in the top panel reveals peaks of unreacted 2-ME eluting at 2 min, the thiol methyltransferase product MTE eluting at 5 min, and a small but measurable amount of the thioether methyltransferase product DMTE eluting at 10.5 min. The peak eluting at 2 min, labeled 2-ME, may also contain polar metabolites of 2-ME other than the methylated products. The lower panel shows the strong inhibitory effect of Adox on MTE and DMTE syntheses in mice. Adox inhibits AdoHcy hydrolase [22] and causes large increases in tissue

concentrations of AdoHcy [14, 17], a product inhibitor of AdoMet-dependent methyltransferases. The nearly complete inhibition by Adox of MTE and DMTE syntheses indicates that these two compounds are produced *in vivo* by AdoMet-dependent methyltransferases.

A quantitative summary of urinary excretion of MTE and DMTE and the effects of Adox is shown in Table 1. Treatment of mice with a single dose of Adox (80 μmol/kg) prior to 2-ME injection completely inhibited the appearance of DMTE in the urine. Adox inhibition of urinary MTE excretion was less complete but still averaged more than 80%. The small amount of [1,2-^{14}C]MTE excreted by the Adox-treated mice may have been synthesized in the latter part of the 24-hr period when the effects of Adox were waning. The majority of urinary radioactivity observed in the 24 hr following treatment with Adox plus 2-ME in mice was attributed to [1,2-^{14}C]2-ME. There was no statistically significant effect on the total ^{14}C excreted upon Adox treatment, although sample sizes were small in these studies. The effects of Adox on the 24-hr urinary excretion by mice of AdoMet and AdoHcy are also shown in Table 1. The present experiments, as well as those described previously [17], demonstrate the presence of AdoMet and AdoHcy in urine of untreated mice and their increase upon Adox treatment. In the present study, Adox induced a 175-fold increase in urinary excretion of AdoHcy. Urinary AdoMet excretion was also increased, probably as a secondary result of tissue AdoMet accumulation caused by AdoHcy inhibition of methyltransferases. Increased levels of urinary AdoMet and AdoHcy resulting from Adox inhibition of AdoHcy hydrolase support previous findings that this phenomenon provides a convenient and noninvasive method of assessing the methylation potential in mice [17].

Sequential methylation in vitro *of 2-ME to MTE and DMTE.* The sequence of reactions shown in Fig. 1 was proven above to occur *in vivo*, but the identity of the enzymes involved (other than their AdoMet dependence) was not revealed by these experiments. To test the involvement of thiol and thioether methyltransferases, a sequential methylation system was reconstructed *in vitro* using $C_{12}E_{10}$-solubilized mouse liver microsomes as a source of thiol methyltransferase along with homogeneous thioether methyltransferase purified from mouse lung. The sequential actions of the two methyltransferases were assessed by measuring either the ability of [*methyl*-^{3}H]AdoMet to act as a methyl donor to unlabeled 2-ME (Experiment A) or the ability of [1,2-^{14}C]2-ME to accept methyl groups from unlabeled AdoMet (Experiment B). The products, MTE and DMTE, were separated by HPLC Method 1 and collected for counting. Figure 3 shows representative HPLC profiles for Experiment A (top panel) and Experiment B (bottom panel). Both types of experiments yielded labeled MTE and DMTE only if thiol and thioether methyltransferases were both present in the reaction mixtures. Figure 4 shows a quantitative summary of several such experiments.

The omission of thioether methyltransferase from

Table 1. Sequential methylation of 2-ME *in vivo* and its inhibition by ADOX

Treatment*	24-hr Urine volume (mL)	Urinary excretion (% of dose/24 hr)				Urinary excretion (nmol/24 hr)	
		Total ^{14}C	[1,2-^{14}C]2-ME	[1,2-^{14}C]MTE	[1,2-^{14}C]DMTE	AdoMet	AdoHcy
Control	0.88	NA†	NA	NA	NA	39.1 ± 5.9^{d}	19.2 ± 2.3^{f}
[1,2-^{14}C]2-ME	1.11	34.6 ± 3.2	19.2 ± 3.1^{a}	11.4 ± 1.1^{b}	1.5 ± 0.4^{c}	46.6 ± 7.1^{e}	26.6 ± 7.2^{g}
Adox + [1,2-^{14}C]2-ME	1.05	30.4 ± 2.7	29.7 ± 3.2^{a}	2.03 ± 0.6^{b}	0.0 ± 0.1^{c}	115 ± 10.3^{d,e}	4569 ± 123^{f,g}

* Three mice in each group were injected i.p. as described under Materials and Methods. Doses: [1,2-^{14}C]2-ME, 450 μmol/kg; Adox, 80 μmol/kg. Values are means ± SEM. Pairs of data with the same superscript letter are significantly different by Student's *t*-test ($P < 0.05$).

† Not applicable since this group was not injected with [1,2-^{14}C]2-ME.

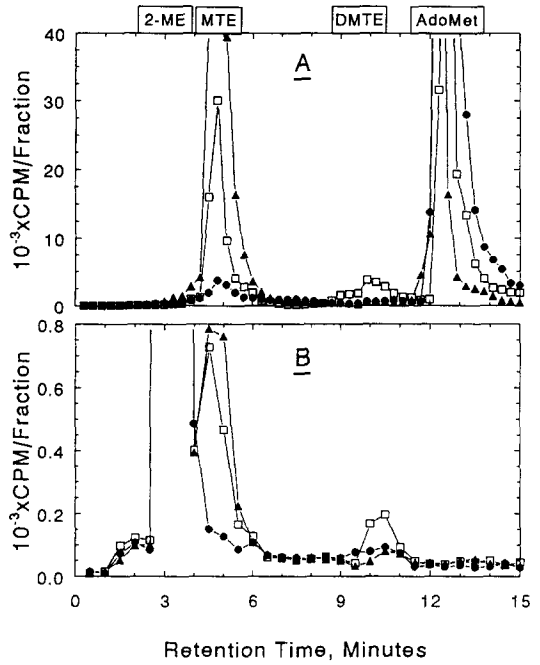

Fig. 3. HPLC elution profiles of sequential reaction mixtures. *In vitro* reconstitution assays were performed as described in Materials and Methods. Reaction mixtures were fractionated by HPLC Method 1. Experiment A (top panel): [*methyl*-^{3}H]AdoMet was used as the methyl donor with unlabeled 2-ME as the acceptor. Experiment B (bottom panel): [1,2-^{14}C]2-ME was used as the methyl acceptor with unlabeled AdoMet. Symbols: complete reaction with both methyltransferases present (□); thiol methyltransferase only (▲); and thioether methyltransferase only (●).

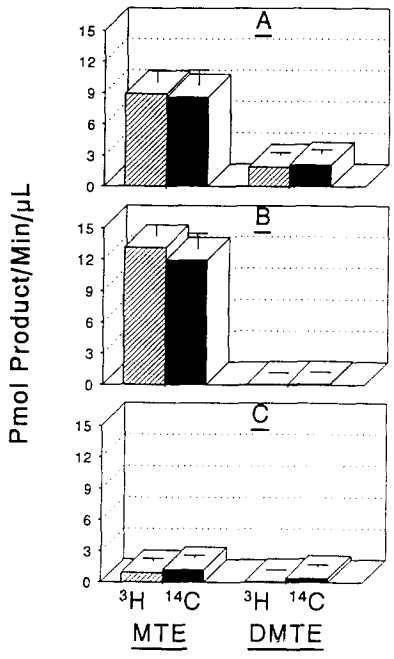

Fig. 4. Sequential methylation *in vitro* by thiol methyltransferase and thioether methyltransferase. *In vitro* methylation assays were performed as described in Materials and Methods and as shown in Fig. 3. The rates of synthesis of MTE and DMTE (pmol/min/μL) were measured using either [*methyl*-^{3}H]AdoMet (bars labeled ^{3}H) or [1,2-^{14}C]2-ME (bars labeled ^{14}C). Values are means ± SEM for triplicate analyses. (A) Complete reaction with both methyltransferases present. (B) Thiol methyltransferase only. (C) Thioether methyltransferase only.

the reaction mixture resulted in a decrease in DMTE synthesis and a greater accumulation of MTE (Figs. 3 and 4). This is consistent with sequential methylation since the thiol methyltransferase product MTE is not depleted by methylation to DMTE due to the absence of thioether methyltransferase. This supports our previous suggestion that more than one methyltransferase is responsible for the sequential methylation of a thiol to its respective dimethyl sulfonium ion [6]. Although 2-ME is not a substrate for thioether methyltransferase [6], a small degree of nonenzymatic transmethylation from AdoMet to MTE apparently occurred, as found by others [16, 18, 19]. In the presence of thioether methyltransferase, this small amount of MTE was converted to DMTE. However, compared with results obtained from the complete reaction, the reaction with thioether methyltransferase alone yielded less than 5% DMTE, and this phenomenon was most evident when [*methyl*-^{3}H]AdoMet was used (Fig. 4). This report definitively demonstrates the requirement for both thiol and thioether methyltransferases for the production of dimethyl sulfonium metabolites from xenobiotic thiols.

DISCUSSION

The results of these studies provide evidence for the occurrence of sequential methylation of a thiol to its respective dimethyl sulfonium ion *in vivo* and *in vitro*. Previous studies have shown that thiol methyltransferase methylates 2-ME and many other thiols *in vitro* [5, 7–9, 12, 13, 16, 18, 19]. Our laboratory has shown previously that MTE is a substrate for thioether methyltransferase *in vitro* [6] and that mice treated with MTE excrete DMTE in their urine [3]. Since the separate enzymic methylations by thiol and thioether methyltransferases have each been considered to be detoxification reactions, it was of interest to determine if their functions were linked and if this methylation sequence was physiologically relevant.

Mice treated with radioactive [1,2-^{14}C]2-ME excreted a substantial amount of [1,2-^{14}C]MTE and a small, but consistently measurable, amount of [1,2-^{14}C]DMTE, proving that 2-ME is methylated to MTE, and that this product thioether is, in turn, methylated to DMTE. This does not identify the methyltransferases involved, but additional *in vivo* experiments using Adox inhibition showed that this sequential methylation occurred in an AdoMet-dependent fashion. Adox has been demonstrated to be a potent inhibitor of AdoHcy hydrolase *in vitro* [22]. When Adox was injected in mice, it increased AdoHcy in tissues [14, 17], which, in turn, inhibited

AdoMet-dependent methylations, such as those producing dimethyl selenide and trimethyl selenonium ion [17] and 2-chloroethyl ethyl methyl sulfonium ion [3]. In the present study, Adox caused MTE to decrease 85%, DMTE to disappear completely, and AdoHcy to increase 175-fold in the urine of mice treated with radioactive 2-ME. This strongly supports the hypothesis that MTE and DMTE are enzymatically synthesized *in vivo* by AdoMet-dependent methyltransferases.

In regard to the thiol compound used for these *in vivo* methylation experiments, 2-ME may not have been the best choice theoretically, since it is considerably hydrophilic and so, as demonstrated herein, it is excreted unchanged at high levels in the urine along with its highly water-soluble product MTE. A more hydrophobic thiol might have a stronger dependence on conversion to the dimethyl sulfonium ion before mobilization from tissues and urinary excretion, thereby giving a higher ratio of dimethyl sulfonium ion to methyl thioether product than did 2-ME. However, 2-ME is the only thiol commercially available in radioactive form and has been thoroughly characterized as a substrate for thiol methyltransferase, which were the reasons for its use in this work.

Since the above experiments suggested that one or more AdoMet-dependent methyltransferases were involved in the sequential methylation, we next tested for this pathway using a system containing a solubilized preparation of thiol methyltransferase along with purified thioether methyltransferase. The results of these experiments demonstrated enzymic methylation of 2-ME to its respective thioether, MTE, and subsequent methylation of MTE to the dimethyl sulfonium ion, DMTE. Reactions were monitored by either using the methyl donating molecule, [*methyl*-^{3}H]AdoMet, labeled at the methyl group that is transferred or by following the methylation of [1,2-^{14}C]2-ME, the methyl acceptor. Both enzymes were necessary for methylation of 2-ME to DMTE since omission of thioether methyltransferase resulted in the absence of DMTE, while omission of thiol methyltransferase resulted in a reduction in the synthesis of both methylated products. These results confirm the suggestion that *in vivo* methylation of 2-ME is AdoMet-dependent, and strongly indicate that thiol and thioether methyltransferases are the enzymes involved.

This report describes: (1) the sequential methylation of 2-ME to DMTE *in vivo*; (2) the AdoMet dependence of this pathway; and (3) the reconstruction of the sequential pathway *in vitro* using pure thioether methyltransferase and a crude source of thiol methyltransferase. Due to this latter point, a degree of uncertainty remains regarding the enzyme catalyzing the first step, since the detergent extract from microsomes used as the source of thiol methyltransferase undoubtedly contained other enzymes. To fully characterize the sequential methylation system, a purified and characterized preparation of thiol methyltransferase is required. Thiol methyltransferase has proven very difficult to purify, perhaps because it is membrane-bound and unstable when solubilized. The most successful previous protocol involved the solubilization of rat liver microsomes with 0.3% Triton X-100 followed by fractionation with Sepharose 4B [8]. However, this method gave only a 21-fold purification of the enzyme. The nonionic detergent $C_{12}E_{10}$ was employed in the present studies because it solubilizes thiol methyltransferase to a higher degree and in a more stable form than detergents used previously, suggesting that it may permit purification to homogeneity. Such an attempt at purification is currently under way in this laboratory and, if successful, will permit more certain identification of thiol methyltransferase as the enzyme catalyzing the first step in the sequential methylation pathway.

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