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S-methylation of diethyldithiocarbamic acid in rat liver microsomes

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1. Human hepatic thiol methyltransferase (TMT) is a microsomal enzyme known to catalyse the *in vitro* *S*-methylation of diethyldithiocarbamic acid (DDC) to form diethyldithiocarbamic acid methyl ester (methyl DDC). *In vivo* data are needed to investigate further the biotransformation of DDC to methyl DDC. Thus, we have characterized the *in vitro* conversion of DDC to methyl DDC using rat liver microsomes with the ultimate goal of establishing an animal model.

2. Formation of methyl DDC in rat liver microsomes was confirmed by hplc analysis.

3. Rat liver microsomes catalysed methylation of DDC with low and high K_m's of 5 ± 6 and 260 ± 80 μM respectively and with corresponding V_{max}'s of $0{\cdot}09 \pm 0{\cdot}05$ and $0{\cdot}59 \pm 0{\cdot}04$ nmol/min/mg protein.

4. Rat liver TMT activity was maximally inhibited by 57 ± 6% by 1000 μM 2,3-dichloro-α-methylbenzylamine (DCMB), whereas human TMT was completely inhibited. The concentration of half maximal inhibition of rat TMT for DCMB was 2 μM.

5. Incomplete inhibition of rat TMT activity by DCMB suggests a possible alternative pathway of methylation.

Introduction

Disulfiram (DSF), an inhibitor of aldehyde dehydrogenase (ALDH), is used in the treatment of alcoholism. The pathway of DSF metabolism and the chemical species responsible for the irreversible inhibition of ALDH are uncertain. After ingestion, DSF is reduced to diethyldithiocarbamic acid (DDC), which is methylated to form diethyldithiocarbamic acid methyl ester (methyl DDC) (Gessner and Jakubowski 1972, Cobby *et al.* 1977). Methyl DDC undergoes further metabolism eventually yielding diethylthiocarbamic acid sulphoxide, a known active metabolite of DSF, and possibly diethylthiocarbamic acid sulphone, a proposed metabolite (Hart and Faiman 1992, Mays *et al.* 1995). Since the putative active metabolites are methylated, we have examined the role of thiol methyltransferase (TMT) in the methylation of DDC using rat liver microsomes.

Human TMT catalyses the *in vitro* biotransformation of DDC to methyl DDC displaying biphasic kinetics with K_m's of 7·9 and 1500 μM and a V_{max} of 0·58 nmol/min/mg protein for the high-affinity component (Glauser *et al.* 1993a). Thiopurine methyltransferase (TPMT), another enzyme known to catalyse the *in vitro* methylation of DDC in man, does not display biphasic kinetics, has a K_m of 95 μM and a V_{max} of 0·053 nmol/min/mg protein (Glauser *et al.* 1993a, Pacifici *et al.* 1993). Comparison of $V_{max}:K_m$ ratios of 4·43 and 0·03 for TMT and TPMT respectively shows that TMT is more efficient and thus more likely responsible for catalysing the methylation of DDC (Glauser *et al.* 1993a). In addition, TPMT preferentially catalyses the methylation of aromatic and heterocyclic sulphhydryls,

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is cytosolic and is sensitive to inhibition by benzoic acid derivatives (Woodson *et al.* 1983). TMT is microsomal, catalyses the methylation of aliphatic sulphhydryls such as DDC and is sensitive to inhibition by 2,3-dichloro-α-methylbenzylamine (DCMB) (Glauser *et al.* 1993a, b). Thus, the reported characteristics of these two methyltransferases suggest that TMT catalyses the *in vivo* methylation of DDC. To evaluate the potential role of TMT in the *in vivo* methylation of DDC, we have characterized rat liver microsomal TMT activity and examined the inhibition of DDC methylation by DCMB.

Materials and methods

Tissue preparation

Livers were obtained from male Sprague-Dawley (Harlan, Indianapolis, IN, USA) rats weighing approximately 250 g. Immediately following removal, the livers were washed twice using ice-cold buffer H (0·7 M sucrose, 0·21 M D-mannitol, 0·002 M HEPES and 0·05 % (w/v) bovine serum albumin), minced and suspended in buffer H to a final concentration of 30 % (v/v). The mixture was homogenized (Sargent Lightnin Model L, Rochester, NY, USA) at 1500 rpm for four up and down cycles using a 30-ml Potter–Elvenhjem homogenizer, diluted to 10 % (v/v) in buffer H and centrifuged (Sorvall RC-5B, Wilmington, DE, USA) at 660 *g* for 15 min at 4°C. Following centrifugation, the resulting pellet was discarded and the supernatant was centrifuged for an additional 15 min at 6800 *g*. The pellet was discarded and the supernatant centrifuged for 1 h at 101 000 *g* at 4°C (Sorvall OTD80B ultracentrifuge, Wilmington, DE, USA). The supernatant was discarded and the pellet suspended in 5 mM TRIS, 1·5 (w/v) KCL (pH 7·8) and centrifuged at 101 000 *g* for 1 h at 4°C. The microsomal pellet was re-suspended in 5 mM TRIS, 1·15 (w/v) KCl and immediately frozen at −80°C in 0·25 or 0·50-ml portions. Human liver microsomes, a gift from R. Weinshilboum, had been harvested from liver biopsies and prepared similarly to rat liver microsomes (Glauser *et al.* 1993a). The concentration of microsomal protein was determined using a Pierce Kinetic BCA assay adapted for a microplate reader.

Chemicals

DDC (lot S-287) was obtained from Fischer Scientific. DCMB (lot VB-B-59) was supplied by Research Biochemicals Inc. (Natick, MA, USA). *S*-(methyl-^{14}C)-adenosyl-L-methionine (Ado-Met), 99·9 % pure in nine parts 10 mM sulphuric acid (pH 2·0) and 1 part ethanol, specific activity 59·3 mCi/mmol (lot 3152084) was obtained from New England Nuclear (Boston, MA, USA) and stored at −20°C. *S*-adenosyl-L-methionine chloride salt (lot 24H7005) for dilution of radioactive Ado-Met was purchased from Sigma (St Louis, MO, USA) and stored at −20°C.

Thiol methyltransferase (TMT) assay

TMT activity was measured by a modification of the method described by Weinshilboum *et al.* (1979). The assay measures the transfer of C^{14}-methyl groups from Ado-Met to DDC. Initially, three blanks were used for each TMT assay. Blank A was enzyme and substrate free, blank B was substrate free and blank C used heat-treated enzymes (Weinshilboum *et al.* 1979). Only blank B had radioactivity above background and was used to correct total counts in further experiments (Glauser *et al.* 1992). The incubations for the TMT assay contained 40 μg rat hepatic microsomes in 100 μl 5 mM Tris buffer, 1·15 (w/v) KCl (pH 7·8), 25 μl DDC solution at various concentrations in 0·6 M phosphate buffer (pH 7·6) and 25 μl 720 mM Ado-Met (specific activity 4·0 μCi/μmol) in a total incubation volume of 150 μl. The reaction was started by the addition of Ado-Met and after an appropriate incubation period at 37°C, the reaction was terminated by the addition of 100 μl N HCl and 10 s of vortexing. Toluene (2·5 ml) was added to the sample followed by 1 min of pulse-vortexing (Glas-Col multi-pulse vortexer, Terre Haute, IN, USA) to extract the non-polar methyl DDC into the organic phase. Samples were centrifuged (IEC model K centrifuge, Needham Hts, MA, USA) for 10 min at 400 *g*. A 1·5-ml portion of the organic layer was removed and added to 4 ml liquid scintillation cocktail (Biosafe II, Research Products Int., Prospect, IL, USA). Radioactivity was counted in a Tri-Carb Liquid Scintillation Analyzer Model 1900TR (Packard, Meriden, CT, USA) for 5 min. Human liver TMT assays were performed using a procedure identical to the rat liver TMT assays.

DCMB was added to appropriate incubations in 25 μl 5 mM TRIS buffer (pH 7·8), 1·15 (w/v) KCl. The volume and molarity were kept constant in assays containing inhibitor by diluting the microsomes in 75 μl instead of 100 μl 5 mM Tris, 1·15 (w/v) KCl to compensate for the 25 μl of DCMB solution added to the incubation.

In experiments using hplc to investigate assay specificity, methyl DDC was extracted using hexane (400 μl) instead of toluene (2·5 ml). Hexane was compatible with direct injection onto the hplc column, which avoided the loss of analyte that occurred during evaporation of toluene. The activity of rat liver microsomes, measured by liquid scintillation assay, was similar for hexane and toluene.

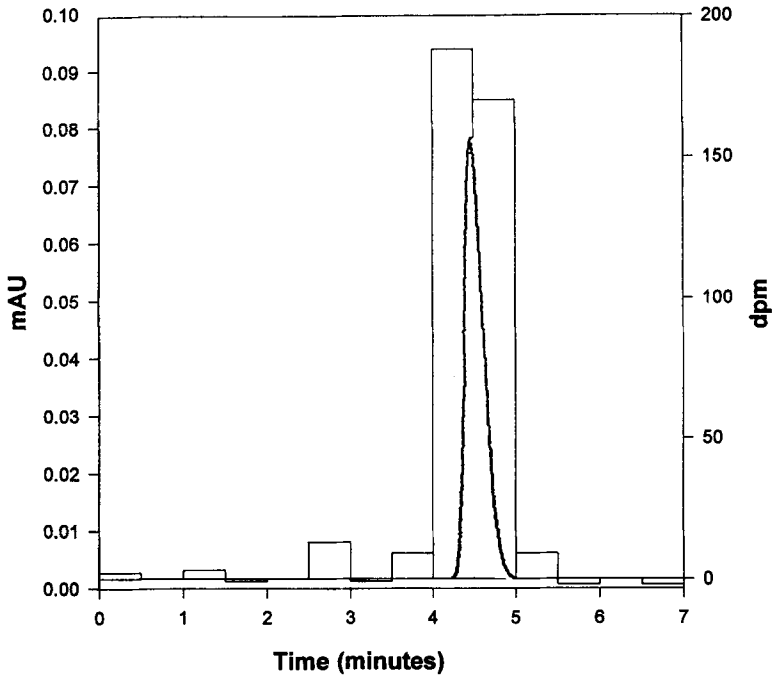

Figure 1. Confirmation of methyl DDC formation. Methylated product extracted from the TMT assay was spiked with 1·6 nmol authentic methyl DDC in 40 μl hexane. This solution (40 μl) was injected directly onto the hplc column for analysis. Fractions of eluant were collected at 30-s intervals and analysed by liquid scintillation. Data show that 96 ± 8% of injected radioactivity eluted as a single peak with methyl DDC standard.

High-performance liquid chromatography

Hplc was used to confirm the formation of methyl DDC using rat liver microsomes in the TMT assay. Instrumentation for hplc analysis consisted of a Waters model 600 Controller, model 717 Autosampler plus, a model 996 photodiode array detector, a C-18 reverse phase, Microsorb Short-One column (80–200 packed with 3 μm particles; 4·6 × 100 mm; Rainin, Woburn, MA, USA) and 37% water/63% methanol mobile phase at a flow rate of 1 ml/min. Methyl DDC standard was prepared using 50 mM methyl DDC in MeOH, diluting it in ethanol (4 mM) and preparing a solution of 1·6 nmol methyl DDC in 40 μl hexane. UV absorbance was monitored at 277 nm, the wavelength maximum for methyl DDC. Hplc eluant was collected at 30-s intervals using a fraction collector (LKB FRAC 200, Pharmacia, Piscataway, NJ, USA). Each fraction was mixed with 4 ml liquid scintillation cocktail (Biosafe II) and radioactivity was measured for 5 min using a Packard Tri-Carb Liquid Scintillation Analyzer Model 1900TR.

Calculations

Initially the kinetic data were analysed using the Eadie–Hofstee equation. Owing to the non-linear nature of DDC methylation, K_m and V_{max} were calculated using a non-linear regression model derived from the Michaelis–Menton equation:

$$V_{total} = V_{max1}[S]/(K_{m1}+[S]) + V_{max2}[S]/(K_{m2}+[S]).$$

Where appropriate, data are reported with a standard deviation. Regression analyses were done using Sigma Stat and WinNonlin.

Results

High-performance liquid chromatography

Previous studies have shown *in vitro* formation of methyl DDC using human liver microsomes in the TMT assay (Glauser *et al.* 1993b). Therefore, we wanted to establish that the use of rat liver microsomes in the TMT assay would lead to

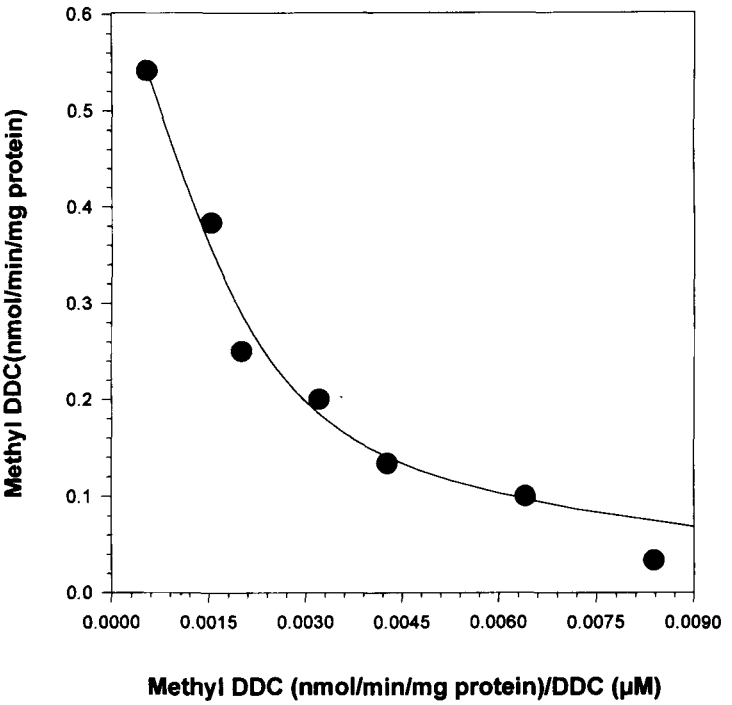

Figure 2. Eadie–Hofstee plot of the methylation of DDC. Each incubation in the assay contained DDC ranging from 3·8 to 1000 μM, rat liver microsomes (40 μg) and Ado-Met (120 mM). Methylation was terminated after 15 min by addition of 1 N HCl. Data points are the means calculated from at least four assays done in triplicate.

formation of methyl DDC. Methylated product, from TMT assays using rat liver microsomes in the incubations, was analysed by hplc. The methylated product from the rat TMT assay coeluted with methyl DDC standard as a single peak at 4·3 ± 0·4 min (figure 1). Additionally, analysis of eluant collected from hplc ($n = 5$) contained 104 ± 9 % of the expected radioactivity. Of that, 96 ± 8 % of the recovered radioactivity eluted in a single peak at the retention time of authentic methyl DDC.

Thiol methyltransferase activity

A series of assays using rat liver microsomes and various concentrations of DDC solution were run to determine the kinetic profile of rat TMT. In addition, the kinetics of DDC methylation for rat TMT and human TMT were compared. As shown in the Eadie–Hofstee plot, *S*-methylation of DDC in the presence of rat liver microsomes displayed biphasic kinetics at DDC concentrations of 3·8–1000 μM (figure 2). Owing to the non-linear nature of the data, the untransformed data were analysed using non-linear regression. Low and high K_m's were calculated and found to be 5 ± 6 and 260 ± 80 μM respectively with corresponding V_{max}'s of 0·09 ± 0·05 and 0·59 ± 0·04 nmol/min/mg protein (figure 3).

Inhibitor studies

DCMB, a known inhibitor of human TMT *in vitro*, was used in assays containing rat liver microsomes to examine its effect upon methylation of DDC. The maximal inhibition of methyl DDC formation was 57 ± 6 % ($n = 8$, with each done

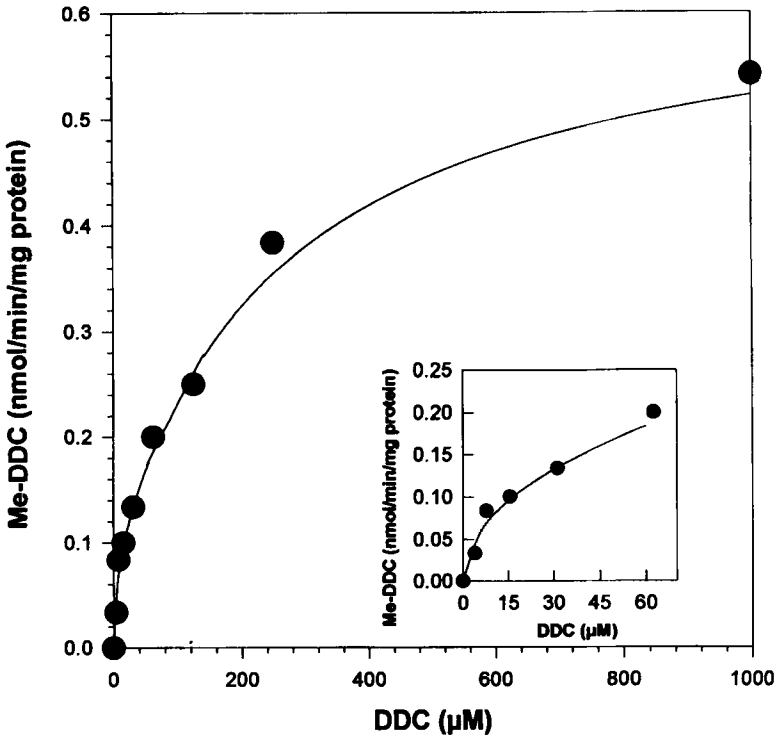

Figure 3. Formation of methyl DDC by rat liver microsomes. Each incubation was done under the conditions stated in figure 2. Data points represent a minimum of four assays done in triplicate. Non-linear regression of untransformed data was used to calculate K_m's of 5 ± 6 and 260 ± 80 μM, and V_{max}'s of $0{\cdot}09 \pm 0{\cdot}05$ and $0{\cdot}59 \pm 0{\cdot}04$ nmol/min/mg protein.

in triplicate) for rat liver microsomes and approximately 100% for human liver microsomes (figure 4). The IC_{50} for inhibition of human TMT by DCMB was approximately 1 μM and the concentration of one-half maximal inhibition of rat liver microsomes by DCMB was approximately 2 μM. The inhibition of methylation by DCMB at pharmacological concentrations of DDC (3·125–31·25 μM) was assessed. Concentrations of DDC near the low K_m (5 ± 6 μM) were inhibited by an average of 48 ± 4% (figure 5). To examine the time dependence of inhibition, rat liver microsomes and DCMB were pre-incubated for 4–30 min. After 4 min or pre-incubation, inhibition of methylation by DCMB had reached approximately 60% and there was no further increase in the observed inhibition with time (figure 6).

We conducted experiments to determine if the incomplete inhibition by DCMB of DDC methylation was due to contamination of the rat liver microsomes with mitochondria, cytosol or both. Each subcellular fraction was substituted for microsomes in the TMT assay. Mitochondria had no activity and cytosol had roughly 10% of the activity seen for microsomes (table 1).

To determine if the incomplete inhibition by DCMB of DDC methylation was inhibitable by another substance, homocysteine was added to the appropriate incubations (Borchardt and Cheng 1978). Homocysteine is a known endogenous inhibitor of Ado-Met-dependent methylation and an ideal candidate to inhibit the Ado-Met-dependent methylation of DDC. Methylation of DDC was completely inhibited by 1 mM homocysteine (table 2). This inhibition was not affected by the addition of DCMB.

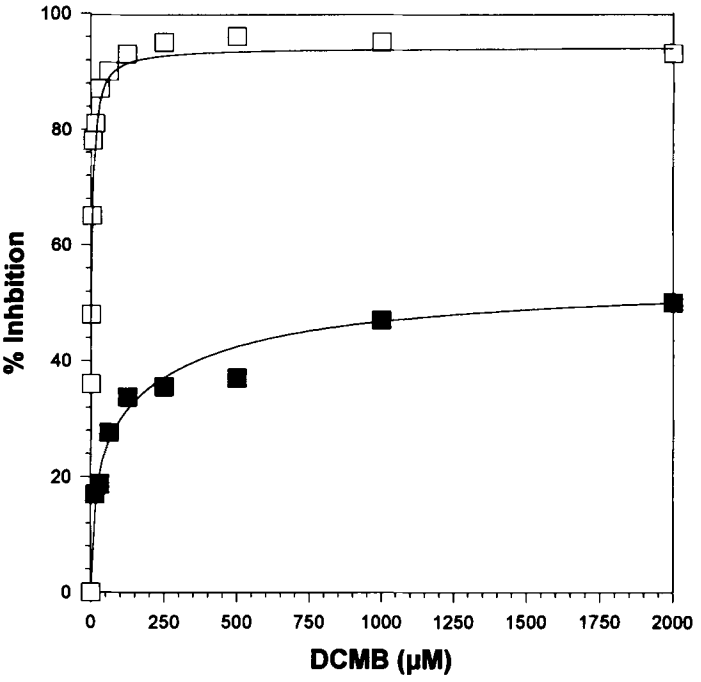

Figure 4. Inhibition by DCMB of DDC methylation by human and rat liver TMT. □, Data for the inhibition by DCMB of human liver TMT; ■, data for the inhibition by DCMB of rat liver TMT. The IC_{50} for DCMB for the inhibition of human TMT is approximately 1 μM. Human liver was inhibitable by 100%. The concentration of half maximal inhibition by DCMB of rat TMT is about 2 μM. DCMB inhibited rat TMT by only 57 ± 6. The data represent at least two assays done in triplicate. DDC concentration is 200 μM. Control activities for the assays were 0·27 (human) and 0·42 (rat) nmol/min/mg protein.

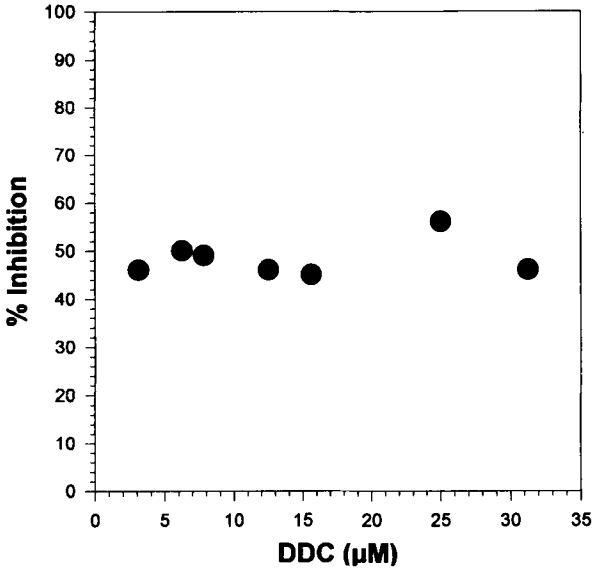

Figure 5. Inhibition of DDC methylation by DCMB at low substrate concentrations. Each incubation contained 40 μg microsomes, DDC (3·1–31 μM) and 1 mM DCMB. Methylation of DDC by TMT was inhibited, on average, by 48 ± 4%. The range of DDC concentrations encompassed the low K_m (5 ± 6 μM).

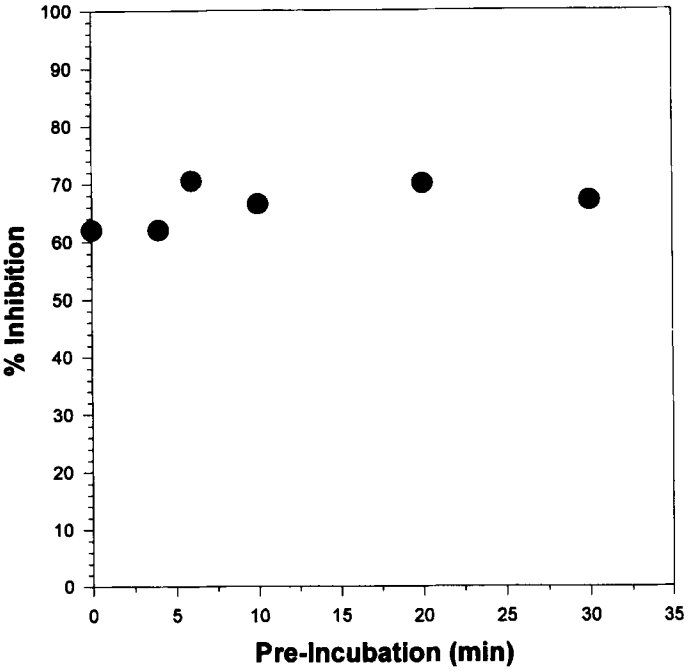

Figure 6. Effect of pre-incubation of DCMB on inhibition of DDC methylation in rat liver microsomes. Microsomes (40 μg) and 1 mM DCMB solution were pre-incubated for 4–30 min after which DDC (200 μM) and Ado-Met (120 mM) were added. Incubations were terminated after 15 min. Data, which represent the mean of two assays done in triplicate, show that by 4 min DCMB had maximally inhibited TMT activity. Control activity was 0·60 nmol/min/mg protein.

Table 1. Subcellular distribution of DDC methylation.

Protein source (μg)		*n*	Specific activity (nmol/min/mg)	% Microsomal activity
Cytosol	40	9	0·08 ± 0·04	11·4
Mitochondria	80	6	nd	—
Microsomes	40	9	0·71 ± 0·17	100

Rat liver mitochondria were prepared using the method of Pedersen *et al.* (1978). Prior to incubation, deoxycholate (0·25 mg/mg protein) was added to the mitochondria. The cytosol was collected during the preparation of microsomes from the supernate obtained following centrifugation at 101 000. nd, Not detectable.

Table 2. Effect of inhibitors on methylation of DDC.

Inhibitor	*n*	% Inhibition
Homocysteine	3	101 ± 1
DCMB	3	50 ± 1
Homocysteine + DCMB	3	102 ± 1

TMT assay used 200 μM DDC, 40 μg rat liver microsomes and 1 mM of the indicated inhibitor (incubation volume = 150 μl).

Control activity = 0·65 nmol/min/mg protein.

Discussion

Thiol methyltransferase plays a significant role in the biotransformation of aliphatic sulphhydryls. *In vitro* human TMT catalyses the transfer of methyl groups from Ado-Met to DDC, an aliphatic sulphhydryl (Weinshilboum *et al.* 1979, Glauser *et al.* 1993a). However, the enzyme(s) involved in the *in vivo* methylation of DDC are uncertain. To delineate further the role of TMT in the *in vivo* methylation of DDC DCMB, an *in vitro* inhibitor of human TMT could be a useful tool. Therefore, assays using rat liver microsomes were performed to characterize further the Ado-Met-dependent methylation of DDC as well as to determine if rat liver TMT activity is sensitive to inhibition by DCMB.

Hplc analysis confirmed that the radiolabelled product from the rat liver microsomal TMT assays was methyl DDC. Under the hplc assay conditions recovery of radioactivity was quantitative and there was no evidence of other significant metabolites. Thus, rat liver microsomes are capable of catalysing the conversion of DDC to methyl DDC *in vitro*.

Rat liver microsomal TMT activity was characterized. The methylation of DDC using rat liver microsomes displayed biphasic kinetics with a low K_m of 5 ± 6 μM and a high K_m of 260 ± 80 μM and corresponding V_{max}'s of $0{\cdot}09 \pm 0{\cdot}05$ and $0{\cdot}59 \pm 0{\cdot}04$ nmol/min/mg protein. Jakoby *et al.* had previously reported a K_m of 12 μM, which is similar to our low K_m of 5 ± 6 μM for the methylation of DDC by rat liver TMT (Weisiger and Jakoby 1979). Although we have suggested that TMT displays biphasic kinetics with more than one K_m, the data are also consistent with one enzyme that behaves allosterically with negative cooperativity. Most enzymes that are allosteric have complex structures with multiple subunits. At this time there is no evidence suggesting that TMT is multimeric. Other investigators have indicated that under experimental conditions similar to those used in our laboratory, TMT has two K_m and V_{max} values. Additionally, they have explained the observation of two K_m's for TMT using Michaelis–Menten kinetics. Given the above, we believe the existence of two K_m's for TMT to be consistent with biphasic kinetics (Glauser *et al.* 1992).

A comparison of our results to the published results obtained by Glauser *et al.* (1993a) shows certain similarities between rat and human liver microsomes. The methylation of DDC using human liver TMT did display biphasic kinetics with a low K_m of 7·9 μM and a high K_m of 1500 μM with a V_{max} of 0·58 nmol/min/mg protein (the V_{max} for the low-affinity component was not reported). These results show that both human and rat hepatic TMT display biphasic kinetics for the methylation of DDC. Additionally, human and rat TMT have similar K_m's for the high-affinity component of the microsomal TMT activity.

It is known that *in vitro* methylation of DDC by human liver microsomes is inhibitable by approximately 100% using 1 mM DCMB (Glauser *et al.* 1993a, b). Therefore, we investigated the sensitivity of rat TMT to DCMB. Experiments using DCMB indicated that methylation of DDC was maximally inhibited by only 57 ± 6%. Additional experiments indicated that the methylation of DDC was inhibited by an average of 48 ± 4% for DDC concentrations ranging from 3·125 to 31·25 μM. This finding indicates that DCMB partially inhibits the ability of TMT to catalyse the methylation of DDC at concentrations of DDC that are of significance *in vivo*. Possible explanations for incomplete inhibition of methylation include contamination of the microsomes with other subcellular fractions or the existence of additional microsomal pathway(s) of methylation in the rat. Our results show that

mitochondrial contamination could not account for the incomplete inhibition of methylation because there was no activity in this fraction. The specific activity in the cytosolic fraction was very low (approximately 10% of the microsomal activity) and therefore could not account for the activity in the microsomes not inhibitable by DCMB. Complete inhibition of DDC methylation by homocysteine is consistent with an Ado-Met-dependent methylation. Taken together, these results imply that the methylation of DDC in rat liver microsomes may have an alternate, Ado-Met-dependent methylation pathway as compared to man *in vitro*. Delineation of the enzyme(s) involved in this process would be of interest.

Acknowledgements

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