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DIETHYLDITHIOCARBAMIC ACID METHYL ESTER
A METABOLITE OF DISULFIRAM*

T. GESSNER and M. JAKUBOWSKI†
Department of Biochemical Pharmacology, State University of New York at Buffalo, Buffalo, N.Y. 14214, U.S.A.

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Abstract—Evidence was obtained of the formation of diethyldithiocarbamic acid methyl ester (DDC-Me) from diethyldithiocarbamate *in vitro* and from disulfiram *in vivo*. The metabolite was identified by two independent methods, that is: (1) by chromatography in three solvent systems, and (2) by isotopic dilution with purification to constant count. Highly active S-adenosyl methionine transmethylase catalyzing methylation of DDC was found in both kidney and liver; the most active transmethylation was associated with the 100,000 *g* microsomal fraction. DDC-[^{35}S]Me was synthesized. Urinary metabolites of DDC-[^{35}S]Me and of DDC-[^{35}S] administered to rats were compared: over 60 per cent of a dose of DDC-[^{35}S]Me was excreted as urinary sulfate in the first 2 days; by contrast, only 16 per cent of a dose of DDC-[^{35}S] was eliminated as sulfate. No diethyldithiocarbamic acid glucuronide was detectable in the urine of DDC-[^{35}S]Me-treated animals; again, by contrast, the glucuronide was the major metabolite of DDC-[^{35}S] (30 per cent of dose). These findings suggest that conversion of DDC-Me to inorganic sulfate occurs without intermediate cleavage to DDC, and therefore point to a pathway to sulfate formation from DDC via its methylated metabolite, DDC-Me.

DITHIOCARBAMATES and related compounds, such as thiuram disulfides, are widely used in industry and agriculture.[1] The compounds are present in rubber products because they serve as antioxidants and as accelerators for crosslinking of rubber. The compounds are used on crops—about seven million pounds of dithiocarbamate fungicides are produced annually in the United States for agricultural use. It is evident therefore that these chemicals can be present in substantial quantities in our environment. Dithiocarbamates have also been used in medicated soap products, but medicinal use of dithiocarbamates is now relegated to the drug known as Antabuse or disulfiram (tetraethylthiuram disulfide), which is employed in the treatment of alcoholism.

Biotransformation of diethyldithiocarbamate (DDC) is especially of interest, since it is the major metabolite of disulfiram and one from which other known metabolites arise, such as carbon disulfide, *N*,*N*-diethylamine, sulfate and diethyldithio-S-glucuronide.[2–5] The known metabolites of DDC, or of disulfiram, do not account for an entire dose of either compound.[4,6] Moreover, observations that some of the effects of disulfiram are delayed and prolonged raise the possibility that an unidentified metabolite of disulfiram may be participating in these effects. For instance, it has been observed that pretreatment with disulfiram is needed for sensitization to ethanol

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† Present address: Institute of Occupational Medicine, Lodz, Teresy 8, Poland.

to occur, and the sensitivity can last for up to 10 days.[7] The more recent observation of Stripp *et al.*[8] regarding the inhibitory effect of disulfiram on drug metabolism is interesting in the context of delayed effects. These workers reported that disulfiram caused a prolongation of hexobarbital sleep time within 2 hr after pretreatment, but decreased drug-metabolizing capacity was observed between 12 and 72 hr after the pretreatment. The workers suggested that their kinetic studies indicate either a qualitative change in the *N*-demethylase system or the presence of a competitive inhibitor (fluctuations in cytochrome P-450 did not explain the effect). It occurred to us that the methyl ester of diethyldithiocarbamic acid could be formed by an enzymatic S-methylation of the dithiocarbamate moiety and that such a metabolite could possibly contribute to some of the observed effects of disulfiram.

Biotransformation of mercaptans to methylthioethers has been reported for a variety of aliphatic thiols.[9,10] S-methylations are also involved in the metabolism of thiopurines, thiopyrimidines,[11–13] and thiophenol.[14] However, ester formation by enzymatic methylation of a thiocarbamic acid has not been reported before; moreover, neither the thiol group of thiocarboxylic acids nor the hydroxyl group of carboxylic acids has been reported to be a methyl acceptor in animal tissues. Hitherto, occurrence of fatty acid methyl esters has been reported in plants.[15] Studies presented here show that formation of a methyl ester, notably methyl ester of diethyldithiocarbamic acid, can also occur in mammals via an enzymatic transfer of a methyl group to the acid moiety. A preliminary report of this work has been given.[16]

MATERIALS AND METHODS

Materials. S-adenosyl-L-methionine iodide (SAM), uridine diphosphate glucuronic acid (UDPGA), and Trizma [Tris (hydroxymethyl) amino methane] were from Sigma Chemical Company, St. Louis, Mo.; S-adenosyl-L-methionine-methyl-[^{14}C] and uridine diphosphate-glucuronic-[^{14}C] acid (D-glucuronic-[^{14}C], uniformly labeled) were from New England Nuclear, Boston, Mass.; unlabeled sodium diethyldithiocarbamate (DDC) was from Fisher Scientific Company; [^{35}S]-labeled sodium diethyldithiocarbamate was purchased from Radiochemical Center, Amersham, Bucks, England. [^{35}S]-labeled tetraethylthiuram disulfide (disulfiram) was prepared by the method of Stromme[17] from the [^{35}S]-diethyldithiocarbamate, the product after three recrystallizations had an m.p. of 72°.

Synthesis of diethyldithiocarbamic acid-[^{35}S]-methyl ester. Unlabeled diethyldithiocarbamic acid methyl ester (DDC-Me) was prepared according to the method of Koch,[18] giving an 80 per cent yield of the product after purification by distillation at reduced pressure. The product was a yellow liquid, b.p. 112–114° at 4 mm mercury, and exhibited in ethanolic solution: λ_1 max. at 251 mμ, ϵ 8,920; λ_2 max. at 277 mμ, ϵ 11,800 (lit. λ_1 max. 252 mμ, ϵ 9,000; λ_2 max. 276 mμ, ϵ 11,500).

The [^{35}S]-labeled compound was prepared by the same procedure. Sodium diethyl-[^{35}S]dithiocarbamate was isotopically diluted with the nonradioactive compound so that 4·14 g (1·6 mc) was used for the synthesis. A methanolic solution (7 ml) of the compound was reacted for 10 min with 1·8 ml methyl iodide, then 7 ml water was added. A yellow liquid of density 1·0977 separated from the reaction mixture. The liquid was distilled as before, giving 2 g (67 per cent yield) of [^{35}S]-labeled diethyldithiocarbamic acid methyl ester (DDC- [^{35}S]Me), b.p. 112–114° at 4 mm mercury.

Paper chromatography. Descending paper chromatography on Whatman No. 1 or 4 was used for separation and identification of metabolites. The following solvent systems were employed: (A) *n*-hexane on paper impregnated with 50% dimethyl formamide solution in ethanol; (B) cyclohexane–*n*-butanol (1:1) on paper impregnated with 50% ethanol solution of dimethyl formamide; (C) *n*-propanol–water (85:15); (D) 70% ethanol on Whatman No. 1.

Appropriate reference compounds were always chromatographed contemporaneously with the extracts under investigation. DDC, DDC-Me and disulfiram were detectable as quenching spots under ultraviolet light. Detection of sulfate was carried out with the aid of barium chloranilate spray according to the method of Bertolini.[19]

Animals and dosing. Male, Blue Spruce mice of HA:ICR strain, about 25 g in weight, and Holtzman rats, about 200 g in weight, were used for the experiments. Labeled diethyldithiocarbamate-[^{35}S] was administered i.v. as an aqueous solution of sodium salt in 0·3 ml (134 μmoles S; 0·88 μc/rat). Diethyldithiocarbamic acid-[^{35}S]methyl ester was injected i.p. with a microsyringe (67 μmoles S; 0·91 μc/rat). The animals were kept in metabolism cages which allowed collection of urine separately from feces. Periodic collections of urine were made; the cages were rinsed and the washings bulked with the urine.* [^{35}S]disulfiram was used as an aqueous suspension stabilized by addition of Tween 80. Five mice were injected i.p. with 0·5 ml suspension containing 15 mg [^{35}S]disulfiram (200 μmoles S; 1·6 μc/mouse). Animals were killed 2 hr after administration of the drug and their livers examined for the presence of diethyldithiocarbamic acid [^{35}S]methyl ester as described below under "Scintillation assay".

Enzyme preparations. For routine assay, freshly excised tissue (liver or kidney) was homogenized with 5 parts of 10% sucrose or 1·15% potassium chloride in a glass homogenizer with Teflon pestle. Subcellular fractionation was carried out as follows: the homogenate was spun for 5 min at 755 *g* to remove cell debris and nuclei; then the supernatant was spun for 20 min at 12,000 *g*; the 12,000 *g* pellet was washed once and respun for 20 min at 12,000 g; the 12,000 *g* supernatant was centrifuged for 60 min at 100,000 *g*. The microsomes were washed once and respun for 60 min at 100,000 *g*. When only the 9000 *g* supernatant was desired, the tissue homogenate was centrifuged for 20 min at 9000 *g*. Particulate fractions were resuspended in sucrose for assays. The fractions used in the enzyme assays possessed approximately equivalent amounts of protein per milliliter of enzyme preparation. Protein content was estimated by the method of Lowry *et al.*[20]

Assay for S-methylation. The enzymatic reaction products in incubations or urine were extracted into aqueous ethanol and assayed as radioactive compounds with the aid of radiochromatography or by scintillation counting.

The enzyme preparations were incubated at 37° for the indicated time intervals. Unless otherwise stated, 10 mM diethyldithiocarbamic acid (sodium salt) was used as substrate and 1 mM (2 μc/ml) S-adenosyl methionine-[^{14}C]methyl (SAM-[^{14}C]) was used as the methyl donor, and the incubations were carried out in 0·2 M phosphate buffer at pH 8·0. The reaction was terminated by addition of 3 vol. of absolute ethanol simultaneously with addition of 5 μl of carrier DDC-Me. After shaking and

* It was deemed unnecessary to maintain urine alkaline for protection of DDC from possible decomposition since Stromme,[4] who took this precaution, found less than 0·1% or urinary ^{35}S to be in the form of DDC after administration of [^{35}S]DDC.

centrifugation at 1000 rev/min for 15 min, aliquots of the supernatants were used for chromatography or for further extraction.

Chromatographic assay. Supernatants were quantitatively applied to paper chromatographic strips and chromatograms were developed in solvent (A), (B), (C) or (D) as indicated. Areas on chromatograms containing radioactive metabolites were located with the aid of a Nuclear Chicago 4 pi chromatogram scanner which was attached to a recorder and to an integrator/printer assembly for summation of counts under each radioactive peak. The presence of the reaction product was established by determining the coincidence of the ultraviolet quenching area due to the carrier DDC-Me with a radioactive peak. Using synthetic DDC-[^{35}S]Me, it was noted that the compound evaporates to a variable extent from chromatograms. This limited the usefulness of the chromatographic assay to qualitative detection of the metabolite and for approximate determinations.

Scintillation assay: This is a quantitative assay for labeled DDC-Me. The method is based on isotopic dilution with unlabeled DDC-Me and extraction of the radioactive metabolite into the oily layer of DDC-Me itself.

In these experiments, the amount of unlabeled DDC-Me used was over a thousand times greater than the expected quantity of the labeled metabolite. Under these conditions, the weight of the metabolite was considered to be negligible in the estimations of the specific activity of thus diluted material. Moreover, the counts present in the isotopically diluted DDC-Me were directly proportional to the per cent conversion of the radioactive precursor to DDC-Me. The counts equivalent to a 100 per cent conversion were determined by counting a sample of the radioactive precursor in the scintillation mixture under conditions (inclusive of the presence of unlabeled DDC-Me) identical to those used for counting of the isotopically diluted metabolite samples. In calculating micromoles of metabolite from the per cent conversion values, it was assumed that 1 mole of labeled DDC-Me arises from 1 mole of DDC-[^{35}S] or from 1 mole of SAM-[^{14}C].

The following procedure was used for the assay: 0·5 ml of the aqueous ethanolic supernatant, obtained as before, was added to 0·5 ml of unlabeled DDC-Me, and 4·5 ml of 25% ethanol was added; the mixture was shaken and centrifuged to separate the aqueous and oily layers. The radioactivity in the DDC-Me layer was assayed by counting a 0·05–0·1 ml portion of this layer in 15 ml of scintillation mixture (which contained 4 g of 2,5-diphenyloxazole (PPO) and 0·5 g of 1,4-bis[2-(5-phenyloxazole)] benzene (POPOP) per liter of toluene) in the presence of 0·5 ml ethanol. We verified by chromatography that labeled DDC-Me was the only radioactive compound which was extracted into the DDC-Me layer from incubations where S-adenosyl methionine-[^{14}C]methyl was the radioactive precursor of the ester. In experiments where DDC-[^{35}S] moiety was labeled, we observed that other [^{35}S] compounds contaminated the extract. It was necessary in such experiments to purify DDC-[^{35}S]Me by microdistillation to constant count. A single distillation achieved the required purification, but constant count was verified by triple distillation.

RESULTS AND DISCUSSION

Identification of DDC-Me as a metabolite of DDC and of disulfiram

As was pointed out in the introduction, biotransformation of thiocarbamates to

methyl esters has not been reported previously. Consequently it was necessary to test in the first instance for the occurrence of such a metabolite. We obtained evidence of formation of DDC-Me from dithiocarbamate *in vitro* and from disulfiram *in vivo*, by two independent methods of identification, i.e. chromatography and isotopic dilution with purification to constant count. In view of the fact that liver and kidney were found to be the most active tissues in a variety of S-methylations[10,13] we examined homogenates of these tissues with respect to the S-methylating activity of DDC.

When mouse liver homogenate prepared in 1·15% KCl was incubated for 1 hr with 1 mM DDC and 1 mM S-adenosyl-methionine-[^{14}C]methyl (SAM-[^{14}C]) in the presence of buffer at pH 8·0, a radioactive metabolite of SAM-[^{14}C] was obtained which behaved on chromatograms like the authentic DDC-Me (Fig. 1b, metabolite M). This metabolite was absent in the control incubation which lacked DDC (Fig. 1a). The metabolite was further examined by chromatography in two other solvent systems consecutively, by a method previously described.[21] Thus, metabolite M separated chromatographically in solvent A was subjected to chromatography in solvent B; again M had the mobility of authentic DDC-Me (Fig. 1c). Subsequent

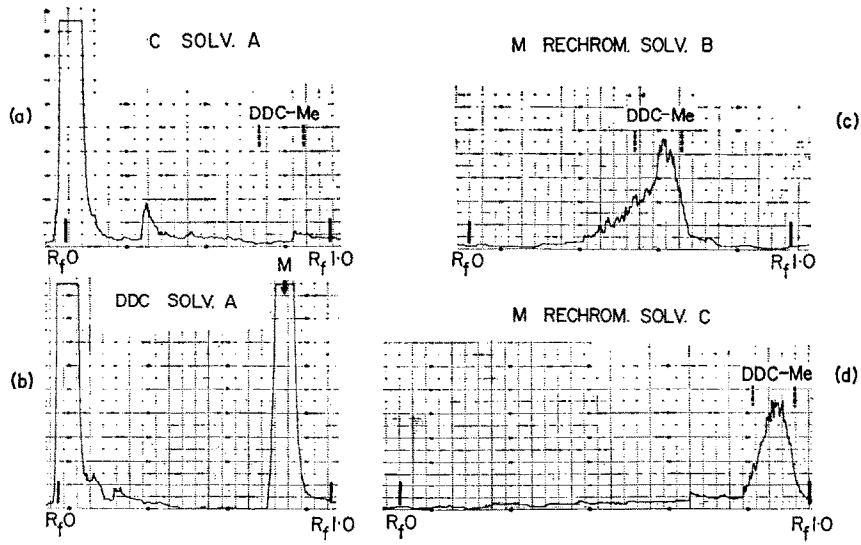

FIG. 1. Chromatographic identification of metabolite M in mouse liver incubations containing diethyldithiocarbamate (DDC) as substrate and S-adenosylmethionine-[^{14}C]methyl (SAM) as the methyl donor. The location of authentic diethyldithiocarbamic acid methyl esters (DDC-Me) is demarcated by broken lines; the locations of the origin and the solvent front are indicated by solid lines. (a) Radiochromatogram of an extract of a control incubation which contained the methyl donor but lacked the DDC substrate. The chromatogram was developed in solvent A, *n*-hexane on paper impregnated with 50% dimethyl formamide solution in ethanol. (b) Radiochromatogram of an extract of an incubation which contained both SAM and DDC. The chromatogram was developed in solvent A. (c) Radiochromatogram of metabolite M in solvent B, cyclohexane–*n*-butanol (1:1) on paper impregnated with 50% dimethyl formamide solution in ethanol. (d) Radiochromatogram of metabolite M in solvent C, *n*-propanol–water (85:15).

chromatography of M in solvent C gave a chromatogram with a single sharp radioactive peak with the R_f value of DDC-Me, thus confirming the identity of the metabolite. A similar experiment was performed on kidney homogenate. Formation of DDC-Me was revealed by chromatography in solvents A and C.

Since disulfiram is readily converted to DDC after absorption,[4] we looked for DDC-Me formed *in vivo* as a metabolite of disulfiram 2 hr after i.p. administration of the ^{35}S-labeled drug. The livers of the treated mice were excised and examined by extraction of DDC-Me and purification to constant count as described under Methods. Small quantities of the metabolite were found. ^{35}S amounting to 0·3 per cent of the dose was extracted from liver into the DDC-Me layer; after purification, the radioactivity associated with the DDC-Me metabolite was found to amount to 0·05 per cent dose, which would account for about 15 per cent of ^{35}S of liver. It is interesting to note that these results fall in line with those that can be calculated from Stromme's[4] data, which show that only 0·5 per cent of the ^{35}S dose was found in rat liver at 2 hr after i.p. injection of [^{35}S]disulfiram, and 10–15 per cent of this radioactivity was in the form of unidentified metabolites.

Microsomal transmethylation of DDC

Remy,[13] while studying S-methylation of thiopurines and thiopyrimidines, observed that mouse kidney was about 6–12 times more active than liver and that the methylation was more active in phosphate than in tris buffer. Accordingly, we examined relative activities of mouse kidney and liver homogenates and compared the effects of the two buffers. It can be seen from Table 1 that liver rather than kidney appears to be more active in S-methylation of DDC, but the effect of the buffers is comparable to that observed by Remy in other S-methylations.

TABLE 1. S-METHYLATION OF DIETHYLDITHIOCARBAMATE BY MOUSE KIDNEY AND LIVER PREPARATIONS*

Buffer	Enzyme source	DDC concn (mM)	Radioactivity of metabolic extract	Amt. metabolized (μmoles/g tissue)
			Counts/min R_f metab.†	
0·1 M Phosphate	Kidney homogenate	None	160	
	Kidney homogenate	1	2110	0·37
	Kidney 9000 *g* supernatant	None	295	
	Kidney 9000 *g* supernatant	1	1214	0·14
			Counts/min in sample‡	
0·2 M Phosphate	Liver homogenate	1	4685	1·05
0·2 M Tris	Liver homogenate	1	2388	0·55
0·2 M Phosphate	Liver homogenate	10	6889	1·55
0·2 M Tris	Liver homogenate	10	4979	1·12

* Tissues were homogenized in 1·15% KCl. Incubations were carried out for 1 hr at 37° at pH 8·0, with 1 mM S-adenosyl-L-methionine-[^{14}C]methyl as the labeled precursor and diethyldithiocarbamate (DDC) as indicated.

† Counted on chromatograms which were developed in *n*-propanol–water (85:15).

‡ Counted as extracts by scintillation assay (see Methods).

Transmethylation of exogenous thiols has been reported to occur in the microsomal fraction of liver for some thiols and in the liver-soluble fraction for others. Thus, Bremer and Greenberg[10] found that rat liver microsomes carried out the methylation of nonphysiological aliphatic thiols, while Remy[13] reported that thiopurines and thiopyrimidines were methylated by enzymes of 100,000 *g* supernatant. It was of interest to examine which transmethylases would act on diethyldithiocarbamate, an amphoteric substrate with both good lipid and water solubility. Our results in Table 2 show that the most active transmethylation of DDC was associated with the microsomal fraction.

TABLE 2. SUBCELLULAR LOCALIZATION OF S-METHYL TRANSFERASE*

Liver fraction	DDC-Me metabolite formed		
	(Counts/min)	(% conversion of SAM-[^{14}C])	(μmoles/ mg protein)
755 *g* supernatant	6900	54	
12,000 *g* particles	2170	17	20×10^{-3}
Microsomes (100,000 *g*)	10,069	78	136×10^{-3}
Soluble (100,000 *g*)	598	4·7	9×10^{-3}

* Mouse liver was homogenized in 10% sucrose. Incubations were carried out for 1 hr at 37° at pH 8·0 in 0·2 M phosphate buffer; 10 mM DDC and 1 mM S-adenosyl-L-methionine-[^{14}C]methyl (SAM-[^{14}C]) were used as substrates. The conversions were determined by scintillation assay as described under Methods.

Further studies of S-methylation of diethyldithiocarbamate were carried out using liver microsomal preparations. The effects of variable concentration of enzyme and substrate were examined, as well as the effect of Tris and phosphate buffers, and the effect of pH. Figure 2a demonstrates the dependence of the reaction on the quantity of microsomal protein. It can be seen that a certain amount of nonenzymatic reaction occurred, as indicated by the intercept on the ordinate; the same amount of methylation was observed when the reaction was carried out in the presence of boiled microsomes. This concurs with the observation of Bremer and Greenberg,[10] who noted that some methylation of hydrogen sulfide and of methyl mercaptan took place in the presence of boiled rat liver microsomes. It can be seen from Fig. 2c that the pH optimum of transmethylation of DDC is at about 8·0. Bremer and Greenberg[10] reported that S-methylation of mercaptoethanol by rat liver microsomes did not exhibit a definite pH optimum, but the reaction rate continued to increase up to at least pH 9·5. On the other hand, Remy[13] observed a pH optimum in the region of 7·4–8·0 and a K_m of $1{\cdot}7 \times 10^{-3}$ M for 2-thiouracil in phosphate buffer. Remy considered that these were the properties of a soluble transmethylase of rat liver, but these particular data were obtained from experiments with 14,000 *g* supernatant, which clearly contained microsomes as well. Perhaps this explains why so much of our data are comparable to those of Remy.[13] We already remarked on the similarity of the results pertaining to the effects of buffers, which showed that S-methylations were about half as extensive in Tris as in phosphate buffer. Figure 2d reveals that this effect has the characteristics of a competitive inhibition. The K_m values for DDC were calculated to be $0{\cdot}77 \times 10^{-3}$ M in phosphate buffer and $1{\cdot}33 \times 10^{-3}$ M in tris buffer.

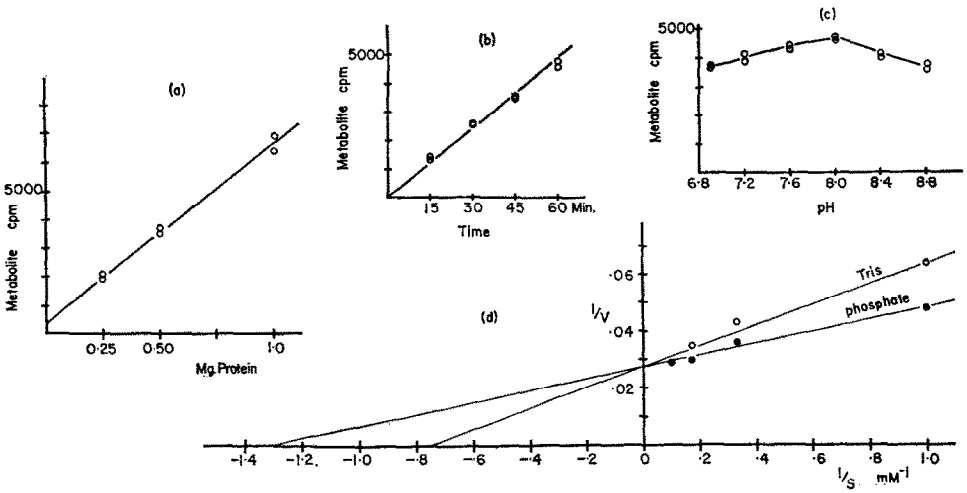

FIG. 2. Properties of mouse liver microsomal S-adenosyl-L-methionine transmethylase. Incubations were carried out at 37° for 1 hr in 0·2 M phosphate buffer at pH 8·0, unless otherwise indicated in the figures; 1 mM S-adenosyl-L-methionine-[^{14}C]methyl (0·8 μc/ml incubation) and 10 mM diethyldithiocarbamate (DDC) were used for all experiments, except where the concentration of DDC was varied. Diethyldithiocarbamic acid methyl ester (DDC-Me) was estimated by the Scintillation assay, as described under Methods. (a) Effect of varying the concentration of microsomal protein on the amount of DDC-Me produced. (b) Effect of varying incubation time on the amount of DDC-Me obtained. (c) Effect of pH on the transmethylation of DDC. (d) Effect of buffer and of variable concentrations of DDC on the amount of DDC-Me obtained.

Metabolic fate of DDC-Me

The experiments aimed to test whether DDC-Me is a compound that is readily metabolized in the animal body and whether an extensive demethylation to DDC takes place. Stromme[4] showed that diethyldithiocarbamate is readily converted to the glucuronic acid conjugate. It appeared likely that if demethylation of DDC-Me takes place readily, then DDC glucuronide should be detectable as a metabolite of DDC-Me. To this end, we incubated mouse liver homogenates with DDC-Me in the presence of labeled UDPGA. Liver homogenate prepared in 1·15% KCl was incubated with 10 mM (2 μc/ml) UDPGA and either 10 mM DDC-Me or 10 mM DDC in 0·1 M phosphate buffer at pH 8·0, for 1 hr at 37°. Extracts of the incubation mixtures were prepared in the usual way and examined by paper chromatography in solvent C, *n*-propanol–water (85:15). Kaslander[3] and Stromme[4] reported that diethyldithiocarbamoyl S-glucuronide has an R_f of about 0·26 in this solvent. Scans of the chromatograms of our extracts revealed that only in the incubation containing DDC was there a metabolite with the R_f of the glucuronide. No radioactive peak with such mobility was detectable in the control incubation which contained UDPGA without DDC or in the incubation which contained DDC-Me. This indicated that no extensive demethylation to DDC had occurred. Subsequent results *in vivo* are consistent with this observation.

Urine of rats treated with DDC-[^{35}S] or DDC-[^{35}S]Me was examined chromatographically in solvent C, and metabolites were detected and estimated by scanning as described under Methods.

Radiochromatograms typical of the experiment are illustrated in Fig. 3. It can be seen that the 0–24 hr urine of a DDC-[^{35}S]-treated animal contains a greater variety of metabolites than the urine of a DDC-[^{35}S]Me-treated animal (Fig. 3, a and c).

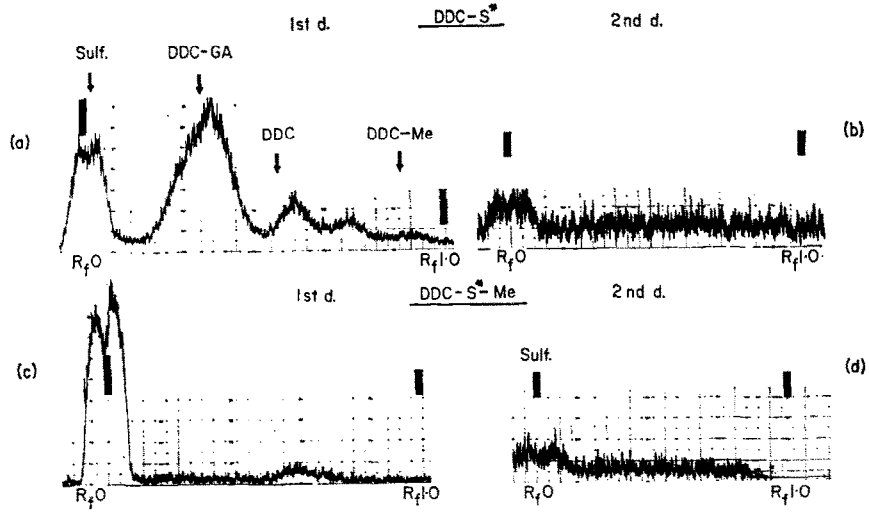

FIG. 3. Chromatograms of urine samples obtained from rats dosed with either [^{35}S]diethyldithiocarbamate (DDC-S*) or with diethyldithiocarbamoyl-[^{35}S]methyl ester (DDC-S*-Me). The chromatograms were developed in *n*-propanol–water (85:15). The positions of: inorganic sulfate (Sulf.) glucuronic acid conjugate of diethyldithiocarbamate (DDC-GA), diethyldithiocarbamate (DDC), diethyldithiocarbamic acid methyl ester (DDC-Me), origin and solvent front are demarcated on the chromatograms. (a, b) Chromatograms of urine samples collected in the first and second day, respectively, after dosing with DDC-S*. (c, d) Chromatograms of urine samples collected in the first and second day, respectively, after dosing with DDC-S*-Me.

Large quantities of inorganic sulfate are detectable in both urine samples, but the metabolite peak with the mobility of DDC glucuronide is seen only in the DDC-[^{35}S]urine. Data in Table 3 show that the glucuronic acid conjugate is the major urinary metabolite of DDC; it accounts for about 30 per cent of the dose. Next is sulfate, which accounts for about 16 per cent of the dose. Small quantities of sulfate continued to be excreted on the second day after administration of DDC-[^{35}S] but no other metabolites were detectable (Fig. 3b). Only 57·2 per cent of the dose was accounted for in terms of urinary excretion (Table 3) up to that time, but it is known that an additional 10 per cent of a dose of DDC is excreted in expired air as carbon disulfide.[4] In the case of DDC-Me, no glucuronic acid conjugate was found (Fig. 3c), but a much higher conversion to sulfate was noted. The latter metabolite accounted for 62–79 per cent of administered DDC-Me (Table 3). The fact that DDC-Me gets extensively converted to sulfate, but does not give rise to DDC glucuronide, shows that conversion to free DDC is not prerequisite to sulfate formation. Our quantitative results rather indicate the opposite, i.e. that S-methylation of DDC may be the major pathway to sulfate formation from DDC. Sarcione and Stutzman,[12] noting that inorganic sulfate was obtained more rapidly from 6-methyl-mercaptopurine

TABLE 3. COMPARISON OF URINARY METABOLITES OF DIETHYLDITHIOCARBAMATE-[^{35}S] AND OF DIETHYLDITHIOCARBAMIC ACID ^{35}S-METHYL ESTER

Compound and dose	Urine sample	Urinary metabolites: Sulfate (% dose)	Glucuronic acid conjugate (% dose)	Other (% dose)	Urine sample (total % dose)
Rat I					
DDC-[^{35}S] 22·7 mg	Day 1	14·1	30·0	11·1	55·2
2·23 μc	Day 2	2·0	neg.	neg.	2·0
Rat II					
DDC-[^{35}S]Me 5·5 mg	Day 1	77·4	neg.	7·6	85·0
0·92 μc	Day 2	2·0	neg.	neg.	2·0
Rat III					
DDC-[^{35}S]Me	0–3 hr	31·4	neg.	4·4	35·8
10·97 mg	3–8 hr	25·5	neg.	2·8	28·3
0·88 μc	8–24 hr	4·8	neg.	0·6	5·4
	Day 1 total	61·7	neg.	7·8	69·5
	Day 2 total	3·5	neg.	neg.	3·5
Rat IV					
DDC-[^{35}S]Me	0–3 hr	33·0	neg.	4·8	37·8
10·97 mg	3–8 hr	18·4	neg.	3·7	22·1
0·88 μc	8–24 hr	6·3	neg.	1·6	7·9
	Day 1 total	57·7	neg.	10·1	67·8
	Day 2 total	4·5	neg.	neg.	4·5

than from 6-mercaptopurine administered to rats, postulated cleavage of methylmercaptan from the S-methyl compound as the pathway to sulfate formation. (Conversion of methyl mercaptan to sulfate has been demonstrated by Cannellakis and Tarver.[22]) Another report consistent with the proposal that thioethers are converted to sulfate via methyl mercaptan is that of Maw,[23] who found that S-methylthioglycollic acid was readily oxidized to sulfate by rat liver slices while, under the same conditions, S-phenyl-thioglycollic acid did not yield any detectable sulfate. Since DDC-Me is an ester, cleavage of methyl mercaptan can be readily envisaged as proceeding with the aid of esterases; this would then be followed by oxidation to sulfate and formaldehyde (Fig. 4). Canellakis and Tarver[22] found that [^{14}C] or methyl mercaptan enters one carbon pool, but [^{35}S] does not get transferred to sulfur-containing amino acids; our proposal would therefore be consistent with this observation.

It was pointed out in the introduction that disulfiram pretreatment caused a decrease in microsomal demethylation of ethyl morphine and that it therefore appeared probable that a metabolite, such as DDC-Me, could be involved in this effect. While the work described here was in progress, Honjo *et al.*[24,25] reported that disulfiram itself and DDC are competitive inhibitors of *N*-demethylation of aminopyrine and of *O*-demethylation of *p*-nitroanisole. DDC-Me can therefore be viewed as one additional potential inhibitor of demethylation. Although our studies show

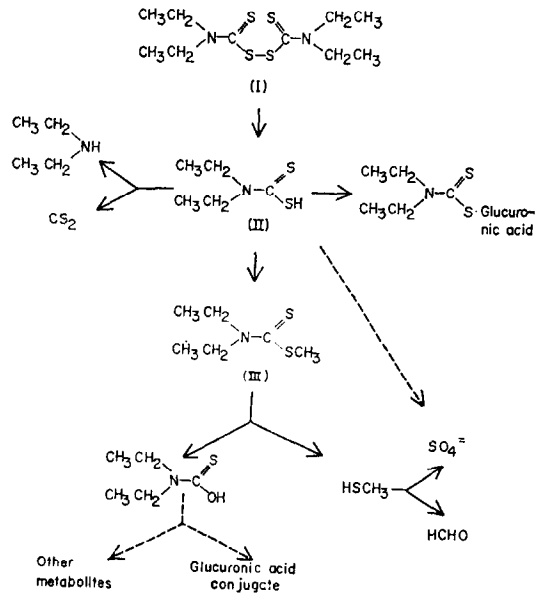

FIG. 4. Metabolic pathways *in vivo* for the sulfur of disulfiram (I), diethyldithiocarbamic acid (II) and diethyldithiocarbamic acid methyl ester (III).

that S-demethylation is not a major pathway for disposition of DDC-Me, this does not rule out the possibility that the compound can present itself as a competitive substrate to the microsomal demethylation system and can undergo a very limited amount of S-demethylation, which was not detectable under our experimental conditions. Moreover, our proposal that DDC-Me is cleaved to yield methyl mercaptan on the path to sulfate formation (Fig. 4) implies that an excellent substrate for S-demethylation would arise in any event. Studies of Mazel *et al.*[26] on microsomal S-demethylations of a variety of S-methyl compounds have shown that methyl mercaptan was the substrate most extensively oxidized to formaldehyde.

Our finding that diethyldithiocarbamate undergoes methylation to methyl ester implies that DDC can interfere with biological methylations by competing for the methyl donor and for transmethylases. It is worth noting that many methylations of endogenous substrates[27] exhibit a pH optimum range of 7·4–8·0 and a K_m of the order of 10^{-4} M, which are comparable values to those we found for DDC. Most methylations of endogenous substrates are carried out by soluble enzymes, but methylation of phospholipids on the pathway to choline formation is a microsomal reaction.[27] We found methylation of DDC to be primarily a microsomal reaction. However, it can be seen from Table 2 that much lower, yet measurable, methylating activity was also associated with the 12,000 *g* particles (mitochondria with lysosomes) and with the soluble fraction. This is a strong indication that DDC can be a substrate of other methylases also. Methylations play an important role in the metabolism of catecholamines and other neurotransmitters. Disulfiram and DDC may be expected to have an effect on these pathways in view of our finding of the DDC-Me metabolite.

The pharmacological properties of DDC-Me are currently under investigation.

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