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S-methylation of dithiocarbamates derived from amino acids

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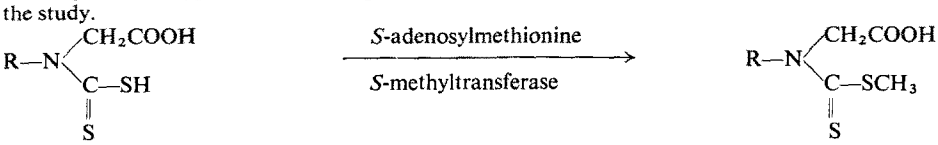
CARBON DISULFIDE is a major occupational health hazard in the viscose rayon industry.[1] Accidental poisoning with carbon disulfide can also occur during its use in pesticide formulations.[2] Toxic effects of carbon disulfide are generally ascribed to the formation of dithiocarbamic acid derivatives which then can complex metal-containing enzymes,[3] but neither the biochemical basis underlying the most prominent pathological changes of chronic exposure nor the metabolism of carbon disulfide and its derivatives is fully understood.[1] Carbon disulfide is known to react with many biochemically important substances[1] including amino acids and proteins,[4] giving dithiocarbamate derivatives. The further fate of these dithiocarbamates has not been fully elucidated. In view of our finding of the diethyldithiocarbamic acid methyl ester as a metabolite of diethyldithiocarbamate,[5] we were interested to find out whether compounds of type I (see below) could be also *S*-methylated in biological systems to compounds of type II. We chose glycine (R—H) and sarcosine (R—CH_3) dithiocarbamates for the study.



(I) (II)

S-adenosyl-L-methionine (methyl-^{14}C), referred to as SAM-^{14}C, was purchased from New England Nuclear, Boston, Mass., and the unlabeled SAM was from Sigma Chemical Co., St. Louis, Mo. The authentic *S*-methyl esters of dithiocarbamate of sarcosine and glycine, which were used as reference compounds for the study, were synthesized as described below.

Synthesis of dithiocarbamate of sarcosine (Sarc. DC) and S-methyl dithiocarbamate of sarcosine (Sarc. DCMe). The barium salt of dithiocarbamate of sarcosine, $[CH_3N(CH_2COOH)C(:S)S^-]_2 Ba^{2+}$, was prepared according to the method of Musil and Irgolic.[6] Sodium salt was generated as needed, as described below under glycine derivative.

The methyl ester of the sarcosine derivative, $CH_3N(CH_2COOH)C(:S)SCH_3$, was prepared according to the method of Rothwell and Wain.[7] The product was recrystallized from a benzene–petroleum ether mixture to give 55 per cent yield of the compound, m.p. 141–144° (lit. 144–146°); ultraviolet spectra show maxima at 248 and 278 nm with ϵ_{248} 7800 and ϵ_{278} 10,000 (in ethanol).

Synthesis of dithiocarbamate of glycine (Gly. DC) and of S-methyl dithiocarbamate of glycine (Gly. DCMe). The barium salt of dithiocarbamate of glycine, $[HN(CH_2COOH)C(:S)S^-]_2Ba^{2+}$, was prepared according to the method of Musil and Irgolic.[6] The sodium salt was then generated as described

below. The methyl ester of the glycine derivative, HN (CH_2COOH)C (:S)SCH_3, was prepared from the sodium salt by reacting it with methyl iodide and was purified as described below.

The barium salt, which was prepared according to the method of Musil and Irgolic,[6] was converted to the sodium salt by addition of a precalculated amount of sodium sulfate and the filtrate was lyophilized. Then 0·031 mole of the product was dissolved in 100 ml of 40% aqueous methanol and reacted with 0·03 mole of methyl iodide. More methanol was added at the end of the reaction to precipitate the inorganic salt. The filtrate was concentrated and the aqueous concentrate lyophilized. The residue of lyophilization was recrystallized several times from ethanol–acetone solutions by addition of diethyl ether. A crystalline product was obtained, m.p. 63–67°, which was analyzed as the sodium salt of the dithiocarbamate derivative, that is HN (CH_2COONa)C (:S)SCH_3. *Anal.* Calc. for $C_4H_6NO_2S_2Na$: C, 25·50; H, 3·20; N, 7·50; S, 34·20; Na, 12·30; Found: C, 25·26; H, 3·62; N, 7·26; S, 33·83; Na, 12·01. Ultraviolet spectra show maxima at 250 and 271 nm with ϵ_{250} 6100 and ϵ_{271} 7870 (in water).

Incubations and metabolite assay. All experiments were done in duplicate. Pooled livers from five mice were used for each experiment. Homogenates of freshly excised livers were prepared in 0·25 M sucrose (20%, w/v) using a glass homogenizer fitted with a 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 100,000 *g* supernatant is referred to as the soluble fraction); the microsomes were washed once and respun for 60 min at 100,000 *g*. A typical 1-ml incubation consisted of the following: enzyme preparation (about 5 mg protein), 0·2 m-mole of phosphate buffer at pH 8·0 (unless otherwise stated), 1 μmole SAM-^{14}C (2 μc), 10 μmoles Gly. DC or Sarc. DC. The incubations were carried out in air in a shaking incubator at 37°. The reactions were stopped by addition of 3 vol. of absolute ethanol containing unlabeled Gly. DC Me or Sarc. DCMe as carrier. The quantity of a metabolite formed was inferred from the amount of SAM-^{14}C converted into the dithiocarbamate methyl ester, assuming a 1 : 1 mole ratio for the transmethylation reaction. The metabolites were located and estimated on paper chromatograms with the aid of a Nuclear Chicago 4 Pi scanner, which was attached to a recorder and to an integrator/printer assembly for summation of counts under each radioactive peak as described earlier.[8] The reference compounds were located as ultraviolet quenching spots. Chromatography was carried out in solvents: (A) *n*-propanol–water (85 : 15), and B *n*-butanol–ethanol–water (5 : 2 : 3). Protein content of fractions was estimated by the method of Lowry *et al.*[9]

The substrates used for the study, Gly. DC and Sarc. DC. as well as the authentic reference compounds for the sought metabolites, Gly.DCMe and Sarc.DCMe, were synthesized as described above.

When mouse liver homogenate was incubated for 1 hr with 10 mM Gly.DC or Sarc.DC and 1 mM 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 in solvent A like the authentic Gly.DCMe (R_f 0·39) or Sarc.DCMe (R_f 0·43). These metabolites were absent in the control incubations which lacked the dithiocarbamate substrates. Moreover, when the metabolite of Gly.DC obtained from a chromatogram in solvent A was subjected to chromatography in solvent B its mobility again coincided with that of authentic Gly.DCMe (R_f 0·43), further confirming the identity of the metabolite. It can be seen from Fig. 1A

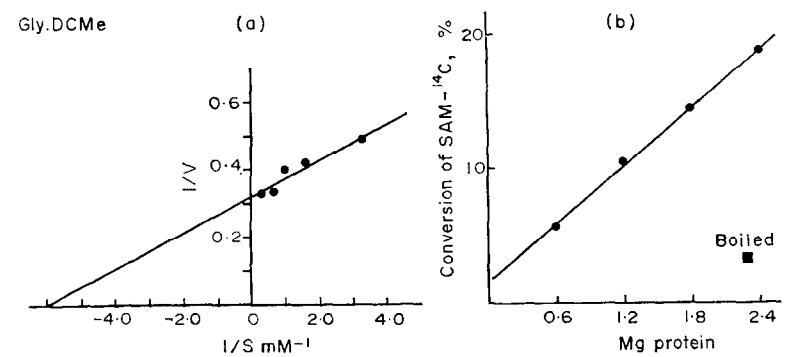


FIG. 1.(a) Effect of variable Gly.DC concentration on the amount of Gly.DCMe obtained in liver homogenate incubations fortified with SAM-^{14}C. (b) Effect of varying the concentration of the soluble fraction on the amount of Gly.DCMe obtained from SAM-^{14}C. The square point shows conversion in the boiled enzyme preparation.

that the methylation of Gly.DC was dependent on the concentration of the substrate; we also observed that, as in the case of other reported *S*-methylations,[10] it was less extensive in Tris than in phosphate buffer.

Next we examined the subcellular location of the transmethylation of the dithiocarbamates of the amino acids. This appeared of interest, since both microsomal *S*-methylations[11] and soluble fraction *S*-methylations[10] have been reported to occur with exogenous thiols. It can be seen from the data in Table 1 that the methylations of Gly.DC and Sarc.DC are mainly soluble enzyme reactions. However, Gly.DC, but not Sarc.DC, appears to undergo some methylation also in the particulate fractions; the significance of this finding cannot be assessed at this time. It is interesting that the dithiocarbamates studied here behaved differently from diethyldithiocarbamate, which we found to be methylated principally by microsomal *S*-methyltransferase at the rate of 135×10^{-3} μmoles/mg of protein/hr.[5,8] However, the latter substrate was also methylated by the soluble fraction to the extent of 9×10^{-3} μmoles/mg of protein/hr,[8] and this is comparable to the reactions reported here for Gly.DC and Sarc.DC (Table 1). These findings suggest that lipid solubility of a substrate may be the factor that determines which *S*-methyl transferase will primarily act upon it.

TABLE 1. SUBCELLULAR LOCATION OF ENZYME(S) *S*-METHYLATING GLY.DC AND SARC.DC*

	Gly.DCMe formed		Sarc.DCMe formed	
Liver fraction	Metabol. peak (cpm)	(μmoles/mg protein)	Metabol. peak (cpm)	(μmoles/mg protein)
755 *g* Supernatant	4942		5069	
12,000 *g* Particles	1827	$3{\cdot}05 \times 10^{-3}$	0	0
Microsomes (100,000 *g*)	2020	$4{\cdot}93 \times 10^{-3}$	0	0
Soluble (100,000 *g*)	6174	$17{\cdot}0 \times 10^{-3}$	5761	$15{\cdot}9 \times 10^{-3}$

Mouse liver was homogenized with five parts of 10% sucrose. Incubations were carried out for 1 hr at 37° at pH 8·0 in 0·2 M phosphate buffer; 10 mM Gly.DC or Sarc.DC (sodium salts) and 1 mM (2 μc/ml) *S*-adenosyl-L-methionine-^{14}C-methyl were used as substrates. The reactions were terminated by addition of 3 vol. of absolute ethanol with simultaneous addition of 200 μg Gly.DCMe or Sarc.DCMe as carrier. After low speed centrifugation, the supernatants were sampled quantitatively for chromatography in solvent A.

A linear relationship can be seen between the soluble enzyme protein concentration and the amount of Gly.DCMe formed (Fig. 1B). The graph shows that a small amount of nonenzymatic *S*-methylation occurs and this is also seen in the boiled enzyme preparation. We examined the pH behavior of the soluble enzyme and found that the amount of metabolite formed continued to increase between pH 6·9 and 8·8 without displaying an optimum. Thus, when mouse liver soluble fraction was incubated, as described in the experimental part, with 0·2 M phosphate buffer of pH 6·9, 7·2, 8·0, 8·4 and 8·8, the conversions obtained were 8·9, 12·5, 13·7, 19, 23·6 and 27·6 nmoles/mg of protein/hr respectively. This behavior is comparable to that seen during *S*-methylation of mercaptoethanol,[11] and contrasts with other *S*-methylations[10] which display pH optima between pH 7·4 and 8·0. An attempt was made to estimate the K_m value for Gly.DC from the double reciprocal plot in Fig. 1A; the calculated value is 0·17 mM. When the data are plotted in the form S/v vs. S, the points fit well a straight line which intersects the S axis at −0·25 mM. It therefore appears that the K_m value for Gly.DC is about 0·2 mM.

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Department of Biochemical Pharmacology,
State University of New York at Buffalo,
Buffalo, N.Y. 14214, *U.S.A.*

M. JAKUBOWSKI*
T. GESSNER

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

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Action of some *N*-methyl derivatives of histamine on salivary and lacrimal secretion of the cat

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IN A previous study one of us[1] investigated the action of a series of histamine derivatives methylated in the side chain amino group, on vascular and extravascular smooth muscle preparations: it was shown that histamine was the most potent compound and that the histaminic activity decreased by increasing the number of methyl groups: taking the activity of histamine as 100 the monomethyl derivative (MMH) was 60–95 per cent as potent and the dimethyl derivative (DMH) 20–55 per cent as potent, the trimethyl derivative (TMH) had a very poor histaminic activity but showed a marked "nicotinic" action. In other investigations it was found that monomethyl and especially dimethyl-histamine, which were found to occur in nature, were actually more active than histamine in stimulating gastric secretion in dogs,[2–4] cats[5] and in the guinea pig.[6]

We wanted to investigate whether other exocrine secretions like salivary and lacrimal secretions behaved as the gastric one or as the smooth muscle.

Experiments were performed on the anaesthetized cat: salivary secretion was measured with a drop counter after cannulation of the submaxillary duct; lacrimal secretion was evaluated by the Schirmer's technique.[7] A simultaneous recording of the systemic blood pressure and respiration was always taken to check continuously the general conditions of the animals.

Compounds used were histamine, the two natural methyl derivatives *N'*-methyl-histamine [4-(2-methylaminoethyl)imidazole] and *N'N'*-dimethyl-histamine [4-(2-dimethylaminoethyl)imidazole], and *N'N'N'*-trimethyl-histamine [4-(2-trimethylaminoethyl)imidazole] which is the quaternary ammonium base of histamine hitherto unknown in nature. Each compound was injected intravenously.

As far as salivary secretion is concerned results are summarized in Fig. 1. It is evident from the figure that mono and dimethyl derivatives behaved exactly as histamine and had quite parallel dose–response curves. Their activities however, were less pronounced than that of the mother substance. The maximum effect was rather low for the three compounds, probably owing to the hypotensive activity which was very remarkable at the highest doses tested (500–750 μg/kg). The behaviour of the trimethyl derivative was quite different and the maximum effect was approximately three times as high as that of histamine. The dose–response curve of this substance paralleled that of nicotine tested for comparison. Nicotine, however, was less active both as regards potency (of which the main index is represented by the threshold dose) and efficacy (of which the main index is represented by the maximum effect).[8] Trimethyl-histamine and especially nicotine caused a remarkable hypertensive effect and a short lasting stimulation of respiratory activity.