Biochemical Studies of Toxic Agents

THE METABOLISM OF IODOMETHANE

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1. The metabolism of iodomethane has been studied after the administration of the compound to rats by subcutaneous injection. 2. The urinary excretion of *S*-methylcysteine, methylmercapturic acid, methylthioacetic acid and *N*-(methylthioacetyl)glycine has been demonstrated by paper chromatography. 3. Methylmercapturic acid and *N*-(methylthioacetyl)glycine have been isolated from the urine of the dosed animals. 4. The methylthio compounds detected represented about 2% of the iodomethane administered.

A number of halogenoalkanes are metabolized in animals to alkylmercapturic acids, i.e. *N*-acetyl-*S*-alkyl-L-cysteines. These include bromo- and iodomethane (Thomson, Maw & Young, 1958; Thomson, Barnsley & Young, 1963), 1-halogenopropanes (Grenby & Young, 1959, 1960) and the 1-bromo derivatives of certain higher alkanes (Bray & James, 1958, 1960; Bray, Caygill, James & Wood, 1964). The excretion of metabolites other than the alkylmercapturic acids has been recognized; ethylmercapturic acid sulphoxide is formed from bromomethane (Thomson & Young, 1960; Barnsley, Thomson & Young, 1964), and unidentified sulphur-containing metabolites are formed from 1-bromobutane and its higher homologues (Bray & James, 1960; James, 1961; Bray *et al.* 1964). *S*-Alkyl-L-cysteines are probably intermediates in the metabolism of the halogenoalkanes (Bray, Franklin & James, 1959; Thomson *et al.* 1963; Barnsley *et al.* 1964), and it has now been shown that the metabolites excreted in the urine of rats after the subcutaneous injection of iodomethane are similar to the metabolites of *S*-methyl-L-cysteine identified by Barnsley (1964*a,b*).

MATERIALS

All melting points and boiling points are uncorrected. Elementary analyses were carried out by Weiler and Strauss, Oxford.

Iodomethane (British Drug Houses Ltd.) was redistilled before use. The preparation of the following compounds was as described by Barnsley (1964*b*). *S*-Methyl-L-cysteine, m.p. 240–245° (decomp.), $[\alpha]_D^{25} -28 \pm 2^\circ$ (c 1 in water) (Found: C, 35.6; H, 6.5; N, 10.5; S, 24.2. Calc. for $C_4H_9NO_2S$: C, 35.5; H, 6.7; N, 10.4; S, 23.7%). Methylmercapturic acid, m.p. 82°, $[\alpha]_D^{25} -33 \pm 2^\circ$ (c 1 in water) (Found: C, 41.0; H, 5.9; N, 8.0; S, 18.4; equiv. 177. Calc. for $C_6H_{11}NO_3S$: C, 40.7; H, 6.3; N, 7.9; S, 18.1%; equiv.

177). Dicyclohexylammonium methylmercapturate, m.p. 181.5–182°, $[\alpha]_D^{25} -9 \pm 1^\circ$ (c 1.5 in water) (Found: C, 60.2; H, 9.3; N, 8.2; S, 9.1. Calc. for $C_{18}H_{34}N_2O_3S$: C, 60.3; H, 9.6; N, 7.8; S, 8.9%). (–)-Methylmercapturic acid sulphoxide, m.p. 140° (decomp.), $[\alpha]_D^{25} -148 \pm 3^\circ$ (c 0.7 in water) (Found: C, 37.7; H, 5.7; N, 7.0; S, 16.3; equiv. 192. Calc. for $C_6H_{11}NO_4S$: C, 37.3; H, 5.7; N, 7.2; S, 16.6%; equiv. 193). (+)-Dicyclohexylammonium methylmercapturate sulphoxide, the salt of (+)-methylmercapturic acid sulphoxide, m.p. 166–167° (decomp.), $[\alpha]_D^{25} +37 \pm 2^\circ$ (c 1.5 in water) (Found: C, 57.2; H, 8.9; N, 7.4; S, 8.7. Calc. for $C_{18}H_{34}N_2O_4S$: C, 57.7; H, 9.2; N, 7.5; S, 8.6%). Methylthioacetic acid, b.p. 126–128°/22 mm. (Found: C, 33.9; H, 6.2; S, 29.6; equiv. 107. Calc. for $C_3H_6O_2S$: C, 33.9; H, 5.7; S, 30.2%; equiv. 106). *N*-(Methylthioacetyl)glycine, m.p. 99.5–100° (Found: C, 37.2; H, 6.0; N, 8.7; S, 19.4; equiv. 163. Calc. for $C_5H_9NO_3S$: C, 36.8; H, 5.6; N, 8.6; S, 19.6%; equiv. 163).

(+)- and (–)-*S*-Methyl-L-cysteine *S*-oxide were prepared by the method of Syngé & Wood (1956). (+)-*S*-Methyl-L-cysteine *S*-oxide, m.p. 160–165° (decomp.), $[\alpha]_D^{25} +125 \pm 5^\circ$ (c 0.4 in water) (Found: C, 31.5; H, 6.1; N, 9.2; S, 21.3. Calc. for $C_4H_9NO_3S$: C, 31.8; H, 6.0; N, 9.3; S, 21.2%). (–)-*S*-Methyl-L-cysteine *S*-oxide, m.p. 165–170° (decomp.), $[\alpha]_D^{25} -110 \pm 2^\circ$ (c 1 in water) (Found: C, 32.3; H, 6.0; N, 9.3; S, 20.7%).

METHODS

Animals and dosing. Male rats were fed on a diet of rat cake [J. Murray and Sons (London) Ltd.] and water, and urine was collected separately from faeces during consecutive 24 hr. periods after dosing. Iodomethane dissolved in arachis oil was injected subcutaneously into the lumbar region of animals which had been lightly anaesthetized with ether.

Chromatography. Paper chromatograms were prepared on Whatman no. 1 paper by the ascending method with two solvents: A, butan-1-ol-acetic acid-water (4:1:5, by vol., top layer); B, butan-1-ol-pyridine-3*N*-NH₃ soln. (4:3:3, by vol.). The platinum reagent of Toennies & Kolb (1951) as modified by Barnsley *et al.* (1964) was used to detect thioethers and sulphoxides, and amino acids were detected by

dipping chromatograms in a solution of ninhydrin in acetone (0.2%, w/v) to which 2.0% of its volume of pyridine had been added immediately before use. The ninhydrin reagent may be used with chromatograms which have previously been treated with the platinum reagent (Barnesley *et al.* 1964).

Determination of thio ethers. The determination of ethylmercapturic acid by reaction with nitroprusside in alkaline solution followed by acidification and the formation of a red colour was reported by Thomson *et al.* (1963). This method has been used to determine methylmercapturic acid and *N*-(methylthioacetyl)glycine, which give colours with extinction maxima at 504 and 496 m μ respectively.

RESULTS

Paper-chromatographic studies

Male rats (body wt. 180–210 g.) were injected subcutaneously with a 10% (w/v) solution of iodo-methane in arachis oil. Each rat received about 50 mg. of iodo-methane/kg. body wt.; doses of twice this value caused a high mortality rate. Urine was collected for successive 24 hr. periods after dosing, and samples which represented 0.1% of the urine excreted by each rat were chromatographed in solvent A. On chromatograms of the urine excreted in the first 24 hr. four metabolites with R_f values 0.29, 0.70, 0.75 and 0.83 (metabolites 1, 2, 3 and 4 respectively) were detected with the platinum reagent, and that with R_f 0.29 was also detected with ninhydrin. The metabolite detected at R_f 0.83 always gave a very weak spot, and occasionally was not detected at all. These metabolites were not detected on chromatograms of the urine excreted in subsequent 24 hr. periods, and were not detected in the urine of rats dosed with arachis oil alone.

Urine (0.2 ml.) was applied as a streak to strips of paper (15 cm. wide), and after chromatography in solvent A each metabolite was eluted with water from the appropriate area of the chromatogram. Because the metabolites with the higher R_f values were poorly separated from each other, it was necessary to elute each metabolite from a horizontal strip cut from the central part of the band of material in order to avoid contamination with the adjacent metabolite. The eluates were evaporated to give a concentration of the metabolite approximately equal to that in the urine, and were subjected to further chromatographic tests (Table 1).

Metabolite 1 appeared to be identical with *S*-methyl-L-cysteine. It was not separated from the synthetic compound on chromatography in solvents A and B, and after oxidation (0.1 ml. of eluate and 0.1 ml. of 3%, w/v, hydrogen peroxide at room temperature for 5 hr.) it gave material identical with *S*-methyl-L-cysteine *S*-oxide. The metabolite appeared to be stable to acid hydrolysis (0.1 ml. of eluate heated in a sealed tube with 0.1 ml. of conc. hydrochloric acid at 95° for 3 hr.), for after chro-

matography in solvents A and B it was revealed by both the platinum reagent and ninhydrin with undiminished intensity, but this demonstration was not unequivocal as traces of other ninhydrin-reacting compounds were present in the eluate.

The behaviour of metabolite 2 corresponded to that of *N*-(methylthioacetyl)glycine. It was not separated from this compound on chromatography in solvents A and B, and after acid hydrolysis under the conditions used for metabolite 1 it gave material identical with methylthioacetic acid. Although a compound which reacted with ninhydrin and had R_f values similar to those of glycine was also detected after hydrolysis, this was occasionally found on chromatograms of hydrolysates of eluates obtained from the corresponding areas of chromatograms of normal urine.

Metabolite 3 corresponded to methylmercapturic acid. Oxidation with hydrogen peroxide and hydrolysis with 6 *N*-hydrochloric acid, as were carried out with metabolite 1, gave methylmercapturic acid sulphoxide and *S*-methyl-L-cysteine respectively.

Metabolite 4 appeared to be identical with methylthioacetic acid on chromatography in solvents A and B, and was stable towards 6 *N*-hydrochloric acid at 95°.

Isolation of methylmercapturic acid and N-(methylthioacetyl)glycine

Fifty-four rats (body wt. 180–210 g.) were each injected with 0.1 ml. of a 10% (w/v) solution of iodo-methane in arachis oil, and the urine excreted in the first 24 hr. after dosing was collected and stored at –25°. The dosing was repeated on two occasions at 4-day intervals. The whole urine collected (2.5 l., pH 7.3) was evaporated at 50° under reduced pressure until the volume was reduced to 170 ml. The concentrated urine was acidified with 30 ml. of conc. hydrochloric acid, and extracted eight times with portions of chloroform-ethanol (4:1, v/v) each equal in volume to that of the urine. The extract was evaporated, the residue was dissolved in 50 ml. of water and the solution was adjusted to pH 5 with 5 *N*-sodium hydroxide. This solution was applied to a column (63 cm. $\times$ 3 cm. diam.) of Amberlite CG-400 resin which had been equilibrated with sodium formate buffer (0.1 *M* with respect to total formate, pH 4.1), and elution was carried out with formate buffer (pH 4.1) of increasing concentration delivered from an apparatus of the type described by Kellie & Wade (1957). The reservoirs were glass cylinders, 43 cm. $\times$ 9.4 cm. diam.; the donor reservoir contained sodium formate (pH 4.1, 0.4 *M* with respect to total formate) and the recipient reservoir initially contained 2 l. of buffer (pH 4.1, 0.1 *M* with respect to total formate). Fractions (25 ml.) were collected and were tested for the presence of thio

others by the reaction with nitroprusside. A red colour was obtained with fractions 87–97 inclusive. These were combined and passed through a column (27 cm. $\times$ 3 cm. diam.) of Zeo-Karb 225 resin (H^+ form) and the resin was washed until the pH rose to 5. The total eluate was evaporated at 50° under reduced pressure and gave a residue which after drying to constant weight over phosphorus pentoxide *in vacuo* weighed 0.12 g. The residue was fractionated on a column (59 cm. $\times$ 3 cm. diam.) of cellulose with butan-1-ol–ethanol–3 *N*-ammonia solution (9:3:1, by vol.), and fractions (25 ml.) were collected. Samples (40 μ l.) of each fraction were spotted on to chromatography paper which was then dipped in the platinum reagent. Fractions 25–41 inclusive (group A) and 44–55 inclusive (group B) gave white spots on the pink background. The fractions in each group were combined and evaporated at 55° under reduced pressure. The residue was dissolved in water and passed through a column (5 cm. $\times$ 1 cm. diam.) of Zeo-Karb 225 resin (H^+ form) and the column was washed with water until the pH of the eluate rose to 5. The total eluate was evaporated and the residue was dried to constant weight over phosphorus pentoxide *in vacuo*.

Methylmercapturic acid (metabolite 3). The residue from group A (35 mg.) was triturated with 5 ml. of acetone, and the acetone solution was filtered. A solution of dicyclohexylamine in acetone (2%, v/v) was added to the filtrate until 1 drop of the solution, when allowed to evaporate on narrow-range

indicator paper, showed an apparent pH 7–8. A white solid (17 mg.) crystallized from the solution, and evaporation gave a further 6 mg. These fractions were combined and dissolved in the minimum of hot ethanol, and were fractionally crystallized by the addition of acetone (5 ml.) followed by light petroleum (b.p. 40–60°) to yield 10 mg. of material.

This product had m.p. 181–182°, which was not depressed on admixture with synthetic dicyclohexylammonium methylmercapturate, $[\alpha]_D^{17} - 8 \pm 2^\circ$ (c 0.9 in water). It gave 98.6% of the theoretical value when assayed by the nitroprusside reagent and had an infrared-absorption spectrum identical with that of the synthetic compound. In chromatographic tests the isolated material behaved identically with synthetic dicyclohexylammonium methylmercapturate, gave methylmercapturic acid sulphoxide on oxidation with hydrogen peroxide, and *S*-methyl-L-cysteine after acid hydrolysis. The results were similar to those given in Table 1.

When the isolation was repeated with the urine of a group of rats that had received a total of 1.35 g. of iodomethane, 9 mg. of material was obtained, m.p. 181.5–182° (Found: N, 7.9; S, 9.7. Calc. for $C_{18}H_{34}N_2O_3S$: N, 7.8; S, 8.9%).

N-(Methylthioacetyl)glycine (metabolite 2). The residue from group B (38 mg.) was triturated with acetone. The mixture was centrifuged and the supernatant was evaporated. The residue was fractionated on a column (60 cm. $\times$ 2 cm. diam.) of cellulose in a way similar to that described above.

Table 1. *Chromatographic examination of the metabolites of iodomethane*

For details see the text. Chromatograms were treated with the platinum reagent and then with ninhydrin.

Solvent ...	R_F					
	A		B		B	
	A	B	A	B	A	B
			Hydrolysed		Oxidized	
Metabolite 1	0.31*	0.32*	0.32*	0.33*	0.19*	0.18*
<i>S</i> -Methyl-L-cysteine	0.32*	0.33*	0.32*	0.33*		
<i>S</i> -Methyl-L-cysteine <i>S</i> -oxide	0.18*	0.19*				
Metabolite 2	0.74†	0.38†	0.86†	0.45†		
			0.18†	0.17†		
<i>N</i> -(Methylthioacetyl)glycine	0.76†	0.39†				
Methylthioacetic acid	0.88†	0.44†				
Glycine	0.16†	0.17†				
Metabolite 3	0.81†	0.46†	0.33*	0.34*	0.40§	0.29§
Methylmercapturic acid	0.82†	0.46†	0.32*	0.34*		
Methylmercapturic acid sulphoxide	0.40§	0.29§				
Metabolite 4	0.88†	0.47†	0.86†	0.47†		
Methylthioacetic acid	0.88†	0.44†	0.88†	0.46†		

* White spot with the platinum reagent and purple with ninhydrin.

† White spot with the platinum reagent.

‡ Purple with ninhydrin.

§ Yellow spot with the platinum reagent.

The crude product isolated from the eluate (26 mg.) was fractionally crystallized by the addition of light petroleum (b.p. 40–60°) to a solution in rather more than the minimum quantity of chloroform–acetone (1:1, v/v), and gave 9 mg. of product, m.p. 99–100°.

This material gave 98.4% of the theoretical value for *N*-(methylthioacetyl)glycine in the reaction with nitroprusside, and on admixture with the synthetic compound its m.p. was unchanged. Its acid equivalent was 165 (calc. 163) and the infrared-absorption spectrum was identical with that of the synthetic compound. In chromatographic tests results similar to those given in Table 1 were obtained. The isolated compound was not separated from synthetic *N*-(methylthioacetyl)glycine on chromatography in solvents A and B, and after acid hydrolysis it gave material chromatographically identical with methylthioacetic acid and glycine in these solvents.

When the isolation was repeated with the urine of a group of rats which had received 1.35 g. of iodomethane, the purest fraction obtained (4 mg.) had m.p. 95–96°. In chromatographic tests it behaved identically with synthetic *N*-(methylthioacetyl)glycine.

DISCUSSION

Some reactions undergone by methylating agents in biological systems have been elucidated in recent

years, and are similar to those of certain of their homologues. The metabolism of dimethylnitrosamine is accompanied by the methylation of the guanine moiety of rat-liver RNA (Farber & Magee, 1960) and the histidine residues in the proteins of rat-liver slices (Magee & Hultin, 1962). Craddock (1964) has presented evidence that methylation of thiol groups occurs in the liver of the rat. Methyl methanesulphonate was stated by Roberts & Warwick (1958) to give rise to metabolites similar to those of its homologue, ethyl methanesulphonate. This has been confirmed by Pillinger, Craig & Jackson (1964), who have reported the presence of methylmercapturic acid in the urine of dosed animals, and have also detected other metabolites, namely methylmercapturic acid sulphoxide and *S*-methylcysteine sulphoxide in the urine and *S*-methylglutathione in the bile.

Bromomethane has been used frequently in studies of the halogenomethanes in biological systems. It methylates histidine residues in wheat protein (Bridges, 1955) and probably reacts with thiol groups, for it inhibits yeast hexokinase (Dixon & Needham, 1946) and other thiol enzymes (Lewis, 1948). It also brings about the depletion of ATP and phosphoglyceric acid in the thoracic tissue of insects, possibly by inhibiting a glutathione-dependent triose phosphate dehydrogenase (Winteringham, Hellyer & McKay, 1958).

Scheme 1. Metabolism of iodomethane. Conversions demonstrated in the present work are shown by continuous lines, and possible metabolic pathways are shown by broken lines.

Iodomethane was chosen for study in the present work, for it is less volatile than bromomethane, of which 24–45% may be eliminated in the expired air of rats after its intraperitoneal injection (Miller & Haggard, 1943). It has been shown by chromatographic studies that after the subcutaneous injection of iodomethane into rats, a number of metabolites similar to those of *S*-methyl-L-cysteine (Barnsley, 1964*a,b*) are excreted in the urine (Scheme 1). They are *S*-methylcysteine (I), methylthioacetic acid (IV), methylmercapturic acid (III) and *N*-(methylthioacetyl)glycine (II), and the two last-named compounds have also been isolated. One pathway for the formation of these various compounds probably involves the production of *S*-methylglutathione (V) as an intermediate, and it has been shown (Booth, Boyland & Sims, 1961; Johnson, 1963) that rat liver contains enzymes which catalyse the reaction of alkyl halides with glutathione. Moreover, it has been found (Foxwell & Young, 1964) that *S*-methylglutathione gives rise to a number of metabolites identical with those of iodomethane. Methylmercapturic acid sulphoxide was not detected as a metabolite of iodomethane, although it has been identified as a metabolite of *S*-methyl-L-cysteine (Barnsley, 1964*a,b*) and of methyl methane-sulphonate (Pillinger *et al.* 1964). Methylmercapturic acid sulphoxide was detected on chromatograms of the urine of dosed animals after the urine had stood for some time at room temperature, but this material was probably an artifact formed by atmospheric oxidation of methylmercapturic acid.

The metabolites identified in the present work were excreted in small amounts which did not appear in chromatographic experiments to be greater than that of the *N*-(methylthioacetyl)glycine, of which a quantity representing 0.5% of the administered iodomethane was isolated. This value is of the same order as was observed by Barnsley (1964*a,b*) for the metabolites of *S*-methyl-L-cysteine, and the low value may be due in part to oxidation of the methylthio compounds to sulphate. Pillinger *et al.* (1964) reported that 20% of the radioactivity administered to rats as ¹⁴C-labelled methyl methane-

sulphonate was excreted in the urine, an amount much greater than the proportion of iodomethane accounted for as methylthio compounds in the present investigation.

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