Formation of Mercapturic Acids

5. METABOLISM OF SOME HALOGENOPARAFFINS AND NITROPARAFFINS*

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The metabolic formation of a purely aliphatic mercapturic acid was first recorded by Roberts & Warwick (1957, 1958), who found that rats to which ethyl methanesulphonate had been administered excreted *N*-acetyl-*S*-ethyl-L-cysteine (ethylmercapturic acid). Thomson, Maw & Young (1958) reported the excretion of ethylmercapturic acid by rats dosed with bromoethane, and Grenby & Young (1959, 1960) showed that *n*-propylmercapturic acid was excreted by rats, rabbits, guinea pigs and mice to which 1-bromo- or 1-iodopropane had been administered. Bray & James (1958) obtained evidence suggesting that 1-bromobutane, -pentane, -hexane, -heptane and -octane, and 1-nitro-ethane and -propane, formed mercapturic acids in the rabbit, and Barnes, James & Wood (1959) showed that a similar reaction occurred with 1-nitrobutane in the rabbit and rat. The evidence was the increased concentration of thiol compounds (as measured by the intensity of the colour given with sodium nitroprusside) observed in the alkali-hydrolysed urines from animals dosed with the bromoalkanes and the chromatographic detection of the mercapturic acids. It was recognized that the nitroprusside reaction was not specific for mercapturic acids and fractionation of the 'apparent mercapturic acid' into ether-soluble (mercapturic acid) and ether-insoluble (sulphur-containing material that was not mercapturic acid) was reported by Bray & James (1960). In the present paper a specific method for the estimation of the aliphatic mercapturic acids is described, and the isolation of some aliphatic mercapturic acids is reported. A preliminary account has been given (James, 1961).

MATERIALS

All melting points are uncorrected. Carbon and hydrogen microanalyses were carried out by F. and E. Pascher, Bonn. Nitrogen was estimated by the Kjeldahl method and sulphur by the flask method (see Macdonald, 1961) and titration with barium perchlorate (Fritz & Yamamura, 1955). The halogenoalkanes except 1-iodohexane were purchased. 1-Nitrobutane was prepared as described by Barnes *et al.* (1959), and 1-nitrohexane, b.p. 181–183°/755 mm. Hg, 99% pure (Br 0.3%), by the same method

from 1-bromohexane. 1-Iodohexane, b.p. 174°/755 mm. Hg, n_D^{20} 1.4933, was prepared by the interaction of 1-bromohexane and sodium iodide in acetone solution and purified by fractional distillation. *N*-Acetyl-*S*-propyl-L-cysteine, m.p. 93–95°, was prepared as described by Grenby & Young (1960). *S*-Butyl-L-cysteine and *N*-acetyl-*S*-butyl-L-cysteine were prepared as described by Bray, Franklin & James (1959). *S*-Pentyl-, *S*-hexyl-, *S*-heptyl- and *S*-octyl-L-cysteine and their *N*-acetyl derivatives were similarly prepared and their properties are listed in Table 1.

METHODS

Paper chromatography. The solvent mixtures used and the R_F values of the reference compounds are given in Table 1. Whatman 3MM paper was used and the chromatograms were developed for about 16 hr. by the descending method. The detecting reagents were the potassium dichromate-silver nitrate reagent of Knight & Young (1958). The ninhydrin-copper nitrate spray of Moffat & Lytle (1959) was used.

Animals, diet and dosage. These were as described by Bray *et al.* (1959).

Determination of metabolites in urine

Bromide. The method of Hunter (1953) was used. In 12 control experiments in which sodium bromide was added to urine the average recovery was 95% (range 84–113%).

Iodide. This was converted into iodine by treatment with sodium nitrite in acid solution and determined by titration with 0.01 *N*-sodium thiosulphate. Allowance was made for the iodine uptake of the acidified urines. A reagent blank with water instead of urine was included in each set of estimations. Under these conditions when sodium iodide was added to urine $91 \pm 9\%$ of the theoretical titration was obtained.

Glucosiduronic acid. The modified naphtharesorcinol method described by Bray, Humphris, Thorpe, White & Wood (1952) was used.

Mercapturic acid. (a) Nitroprusside method. This was used as described by Bray, James & Thorpe (1958) and Barnes *et al.* (1959).

(b) Chromatographic method. This was based on the method of Fisher, Parsons & Morrison (1948) for the determination of amino acids on paper chromatograms. The 24 hr. urine samples were adjusted to pH 6.8 by the addition of 2 *N*-HCl. The volumes were then made up to 300 ml. for rabbit, 30 ml. for rat and 100 ml. for guinea-pig urine. Duplicate samples (usually 0.3 ml.) of the urines (further diluted if necessary to give a suitable concentration) were then applied to strips (3 in. wide) of Whatman 3MM paper, each spot being 1.1 in. from the edge. A solution of the appropriate mercapturic acid in normal

* Part 4: Bray & James (1960).

Table 1. *Properties of alkylcysteines and acetyl derivatives*

Compound	Melting point (decomp.)	$[\alpha]_D^{25}$ ($\pm 4^\circ$) (c 1.0 in 0.1N-KOH) + 24°	N (%)		S (%)		Equiv.		R_F		
			Found	Required	Found	Required	Found	Required	In solvent A	In solvent B	In solvent C
			8.0*	7.9*	18.5*	18.1*	—	—	0.61	0.27	0.66
<i>S</i> -Butyl-L-cysteine	249° (decomp.)										
<i>S</i> -Pentyl-L-cysteine	239 (decomp.)	+9	7.4	7.3	17.1	16.8	—	—	0.66	0.33	0.70
<i>S</i> -Hexyl-L-cysteine	238 (decomp.)	+7	7.1	6.8	16.4	15.6	—	—	0.70	0.39	0.74
<i>S</i> -Heptyl-L-cysteine	218	+6	6.6	6.4	14.6	14.6	—	—	0.75	0.47	0.78
<i>S</i> -Octyl-L-cysteine	227 (decomp.)	+6	6.1	6.0	14.2	13.7	—	—	0.77	0.50	0.81
<i>N</i> -Acetyl- <i>S</i> -butyl-L-cysteine	67*	(c 1.0 in ethanol) -24*	6.6*	6.4*	14.8*	14.6*	204*	219*	0.92	0.39	0.71
<i>N</i> -Acetyl- <i>S</i> -pentyl-L-cysteine	98	-17	6.0	6.0	13.9	13.7	233	233	0.93	0.44	0.75
<i>N</i> -Acetyl- <i>S</i> -hexyl-L-cysteine†	87	-15	5.6	5.6	13.0	13.0	244	247	0.94	0.49	0.79
<i>N</i> -Acetyl- <i>S</i> -heptyl-L-cysteine	83	-16	5.1	5.4	12.9	12.3	272	261	0.95	0.51	0.79
<i>N</i> -Acetyl- <i>S</i> -octyl-L-cysteine	82	-15	5.6	5.2	12.0	11.6	281	275	0.95	0.55	0.82

* Bray *et al.* (1959); $[\alpha]_D$ at 23°.† Found: C, 53.5; H, 8.4. $C_{11}H_{21}NO_3S$ requires C, 53.4; H, 8.5%.

urine of a concentration similar to that of the urine under examination was applied to paper strips in amounts of 20–60 μ g. The samples were delivered from an Agla micro-syringe or a syringe pipette. The urine was dried by a current of warm air after the application of each spot. The strips were all placed in one tank and were developed by the descending method with solvent C for 16 hr. The strips were dried in a current of warm air, sprayed lightly with the potassium dichromate solution followed by silver nitrate solution, and dried at room temperature. The spots were outlined and their areas measured with a planimeter. Over the range used the areas of the spots varied linearly with the concentration. There was some variation in the areas from day to day and a calibration curve was constructed for each set of determinations. The amounts of mercapturic acid present in the urines were read off from the calibration curve. The average recovery in eight control experiments was 99.5% with a maximum error of $\pm 7\%$. No substance reacting with the potassium dichromate-silver nitrate reagent and having an R_F value similar to that of the mercapturic acids or 'second metabolites' was present in normal urine. Determinations were carried out on successive 24 hr. urines until mercapturic acid was no longer detected.

RESULTS

Isolation of metabolites

Isolated metabolites had the same R_F values in all three solvent systems as did the synthetic compounds.

N-Acetyl-S-butyl-L-cysteine from rabbit urine. The 24 hr. urine of 12 rabbits each dosed with 625 mg. of 1-bromobutane was acidified with 11N-hydrochloric acid to pH 1–2 and continuously extracted with ether for 72 hr. The solvent was allowed to evaporate from the extract at room temperature. The residue was dissolved in 60 ml. of solvent B and fractionated on a column of Whatman cellulose powder (25 cm. $\times$ 3.5 cm. diam.) by elution with solvent B and collection of eluates of 20 ml. Samples of each eluate were examined by paper chromatography with solvent B and the Knight & Young (1958) reagent for detection. The eluates which contained butylmercapturic acid were combined and further fractionated on a cellulose column or on paper sheets with the same developing solvent. Butylmercapturic acid was isolated from the purified material as colourless prisms, m.p. 69–71°, after recrystallization from ether–light petroleum (b.p. 40–60°), alone or mixed with an authentic specimen (m.p. 70°). The yield was 2% of the dose.

N-Acetyl-S-pentyl-L-cysteine from rabbit urine. By the same procedure pentylmercapturic acid was isolated from the urine of six rabbits each dosed with 620 mg. of 1-bromopentane. It had m.p. 98°, after recrystallization from ether–light petroleum (b.p. 40–60°), alone or mixed with an authentic specimen. The yield was 0.5% of the dose.

N-Acetyl-S-butyl-L-cysteine from rat urine. The urine from 12 rats that had each received 1-

bromobutane (110 mg.) was acidified and extracted with ether as for rabbit urine. The evaporated extract was extracted with chloroform. The syrup obtained on evaporation of the chloroform from the extract was extracted with water and the aqueous solution concentrated and applied as bands to sheets of Whatman 3MM paper. These were developed with solvent B for 16 hr. The bands corresponding to butylmercapturic acid were excised, cut into small pieces, suspended in 0.1N-hydrochloric acid and continuously extracted with ether. The syrup obtained by removal of ether from the extract crystallized (after addition of a seed of butylmercapturic acid) and was recrystallized from ether–light petroleum (b.p. 40–60°). The crystals (90 mg.; yield 4%) had m.p. 69–71° alone or mixed with an authentic specimen.

N-Acetyl-S-pentyl-L-cysteine from rat urine. By the same procedure the urine of 12 rats each dosed with 92 mg. of 1-bromopentane yielded 32 mg. (1.7% of dose) of pentylmercapturic acid, m.p. 100–101° not depressed by admixture with the synthetic acid (m.p. 98°).

N-Acetyl-S-hexyl-L-cysteine. By the same procedure, except that the separation on paper sheets was repeated, the urine of 12 rats that had each received 90 mg. of 1-bromohexane yielded, after recrystallization from water, 3 mg. (0.2% of dose) of hexylmercapturic acid, m.p. 86° alone or mixed with an authentic specimen (m.p. 87°).

N-Acetyl-S-heptyl-L-cysteine. A syrup was obtained by the same procedure from the urine of 12 rats that had each received 67 mg. of 1-bromoheptane, but no crystals were isolated. Chromatography in solvents A and B indicated the presence of heptylmercapturic acid.

Detection of sulphur-containing metabolites by paper chromatography

Urine samples were applied directly to the paper; in urines in which no mercapturic acid was detected ether extracts of the acidified urines were examined. Papers were developed with solvents A, B and C. Mercapturic acid was readily detected in the urines obtained from rabbits dosed with the compounds listed in Table 3 except 1-nitrobutane, 1-chlorohexane, 1-nitrohexane, 1-bromoheptane and 1-bromo-octane. The ethereal extracts of the acidified urines obtained after dosing with 1-nitrobutane, 1-nitrohexane and 1-bromoheptane contained small amounts of the expected mercapturic acids. Similar results were obtained with the urines of dosed rats except that heptylmercapturic acid was detected directly in urine as well as in the ethereal extracts, and that hexylmercapturic acid was detected in the ethereal extract of the urine of rats dosed with 1-chlorohexane. No mercapturic acid was detected in urines of either rabbits or rats

dosed with 1-bromo-octane. No evidence was obtained either directly or in ethereal extracts for the presence of mercapturic acid in the urines of guinea pigs given 1-bromobutane, 1-nitrobutane or 1-bromohexane. In the urines of rabbits and rats dosed with the compounds listed in Table 3 except 1-bromopropane a second sulphur-containing metabolite was detected but the amount in the urines of rabbits dosed with 1-chlorohexane was very small. These metabolites were separated by adsorption on charcoal (cf. Boyland & Sims, 1958) from the urine of rats dosed with 1-bromobutane, 1-bromopentane, 1-bromohexane, 1-bromoheptane and 1-

bromo-octane. They were purified by chromatography on paper sheets with solvent A and their R_F values are recorded in Table 2. The R_M values (Bate-Smith & Westall, 1950) with those calculated for the *S*-alkylcysteines and their *N*-acetyl derivatives are shown in Fig. 1.

Quantitative results

Excretion of glucosiduronic acid. The average increase, expressed as a percentage of the dose, in glucosiduronic acid excretion by the rabbit dosed with 1-bromobutane and 1-bromopentane was 4, the range in six experiments being 0–9. Corresponding values for 1-bromohexane were 11 (range 0–20 for seven experiments) and for 1-bromoheptane 12 (range 0–22 for six experiments). In three experiments with 1-bromo-octane no increased excretion was observed.

Excretion of mercapturic acid, bromide and iodide. The percentages of the doses excreted are given in Table 3. Analyses of the urine were continued until the concentration of metabolite returned to the 'normal' level, except where otherwise indicated. In experiments not included in Table 3 the analysis of successive urine samples from rabbits and guinea pigs dosed with sodium bromide or the 1-bromoalkanes showed that bromide was slowly excreted over 4 days whereas the excretion of iodide by animals dosed with sodium iodide or the 1-iodoalkanes was usually complete in 12–27 hr.

DISCUSSION

The amounts of mercapturic acid in the urines of rabbits and rats dosed with 1-bromo- or 1-iodoalkanes (Table 3) show that the proportion of the dose converted into mercapturic acid decreases from 1-bromo- or 1-iodo-butane as the series is ascended. The results are supported by the isolation of *N*-acetyl-*S*-butylcysteine and *N*-acetyl-*S*-pentylcysteine from the urines of rabbits and rats dosed with 1-bromobutane and 1-bromopentane and of *N*-acetyl-*S*-hexylcysteine from the urine of rats dosed with 1-bromohexane. The yields of the mercapturic acids isolated and the failure to isolate higher members of the series are such as might be expected from the quantitative results. Grenby & Young (1960) isolated from rat urine about 1% of doses of 1-bromopropane and of 1-iodopropane as propylmercapturic acid but, as they pointed out, the halogenopropanes may be partly lost in expired air so that the percentage of the absorbed dose converted into mercapturic acid was probably higher than 1%. They did not determine the amount of mercapturic acid excreted quantitatively.

The amounts of mercapturic acid found in the urines by the specific chromatographic method are

Table 2. R_F values of 'second metabolite' separated from the urine of rats dosed with 1-bromoalkanes

Compound	R_F		
	In solvent A	In solvent B	In solvent C
1-Bromobutane	0.78	0.12	0.58
1-Bromopentane	0.83	0.15	0.60
1-Bromohexane	0.86	0.20	0.65
1-Bromoheptane	0.89	0.28	0.69
1-Bromo-octane	0.90	0.34	0.74

Fig. 1. R_M values of the mercapturic acids, alkylcysteines and the unidentified sulphur-containing metabolite obtained from urines of rats dosed with 1-bromoalkanes with 4, 5, 6, 7 and 8 carbon atoms/molecule. The letters A, B and C indicate the solvent mixtures the composition of which is given in Table 1. —, Mercapturic acid; - - - -, alkylcysteine; ———, unidentified metabolite.

Table 3. *Excretion of mercapturic acid and halide by animals given halogenoalkanes, nitroalkanes or sodium halides*

Amounts excreted are expressed as percentages of the dose, ranges are given in parentheses and numbers of experiments are indicated by superscript numbers. Unless the times are given, the results are for the amount excreted until the 24 hr. excretion returned to the normal level. N.D. indicates not detected; — indicates not examined. In the guinea pig, the excretion of true mercapturic acid was examined after administration of all the alkanes listed except chlorobutane, chlorohexane and nitrobutane but none was detected. The values for apparent mercapturic acid were 18 (10–12)¹⁴ from bromobutane and 10 (9–12)³ from nitrobutane (doses 1.0 m-mole/kg.).

Compound	Rabbit					Rat				Guinea pig			
	Dose (m-moles/kg.)	Mercapturic acid	Apparent mercapturic acid	Bromide or iodide (96 hr.)	Bromide after 4 days	Iodide (total)	Dose (m-moles/kg.)	Mercapturic acid	Apparent mercapturic acid	Iodide (total)	Dose (m-moles/kg.)	Bromide or iodide (24 hr.)	Iodide (total)
1-Bromopropane	1.9	2.7 (1.3-5.2) ⁹	—	—	—	—	2.3	5.1 (4.7-5.7) ⁶	—	—	1.2	—	—
1-Chlorobutane	1.7	0.5 (0.5) ⁸	12 (9-15) ⁶	—	—	—	3.0	Trace ³	—	—	—	—	—
1-Bromobutane	1.7	4.7 (3.9-5.7) ³	41 (27-54) ²⁰	16 (11-21) ⁶	43 (41, 44) ²	—	2.3	6.3 (4.0-8.1) ⁸	54 (21-72) ¹⁶	—	1.0	13 (9-16) ³	—
1-Iodobutane	0.9	8.9 (6.5-11.7) ⁶	32 (21-39) ¹⁰	43 (27-57) ⁵	—	56 (41-78) ⁷	1.4	22 (17-27) ⁶	—	50 (33-72) ¹⁸	0.7	—	69 (56-82) ³
1-Nitrobutane	1.7	Trace ³	23 (20-28) ⁵	—	—	—	3.3	Trace ³	51 (30-64) ⁶	—	1.0	—	—
1-Bromopentane	1.5	2.3 (1.6-3.7) ⁹	21 (16-23) ³	27 (18-40) ³	50 (49-52) ³	—	2.1	5.9 (2.8-9.0) ⁷	35 (14-55) ⁶	—	1.0	—	—
1-Iodopentane	0.8	3.6 (2.8-4.6) ³	27 (18-37) ⁶	49 (31-63) ⁷	—	74 (59-84) ⁹	1.1	5.7 (5.2-6.4) ³	—	60 (33-95) ⁹	0.5	—	74 (69-78) ³
1-Chlorohexane	1.3	N.D. ³	2 (0.3-3) ³	—	—	—	2.5	0.5 (0.5) ³	19 (18-20) ³	—	—	—	—
1-Bromohexane	1.3	0.9 (0.3-2.3) ⁷	23 (21-25) ³	29 (17-42) ⁴	48 (45-53) ³	—	2.0	3.1 (1.6-5.4) ¹¹	52 (23-69) ⁶	—	0.9	—	—
1-Iodohexane	0.7	0.8 (0.1-2) ⁶	22 (19-23) ³	—	—	46 (16-66) ⁹	1.0	1.4 (1.0-1.6) ⁵	51 (42-67) ³	71 (52-94) ⁴	0.6	—	94 (69-124) ⁶
1-Nitrohexane	1.2	N.D. ³	1 (0-2) ³	—	—	—	2.4	Trace ³	6 (4-7) ³	—	—	—	—
1-Bromoheptane	1.1	N.D. ⁶	16 (12-19) ³	20 (11-22) ⁴	66 (63-71) ³	—	1.9	2.3 (1.0-3.1) ⁶	70 (40-90) ⁶	—	0.8	—	—
1-Iodoheptane	0.7	Trace ³	17 (3-28) ⁶	71 (59-87) ⁵	—	70 (57-83) ³	1.0	N.D. ⁶	—	60 (48-72) ³	0.6	—	79 (69-85) ³
1-Bromo-octane	1.1	N.D. ⁶	12 (0-21) ⁶	19 (17-20) ³	53 (47, 58) ²	—	1.7	N.D. ⁶	46 (24-59) ⁵	—	0.7	—	—
Sodium bromide	1.7	—	—	12 (22, 25) ³	48 (44-55) ³	—	—	—	—	—	2.5	18 (16, 19) ²	—
Sodium iodide	0.8	—	81 (55-100) ³	—	—	86 (56-108) ³	1.4	—	—	77 (51-92) ¹⁵	0.7	59 (50-73) ³	103 (87-115) ³

much lower than those indicated by the non-specific nitroprusside method of Barnes *et al.* (1959). Chromatographic examination of the urines of rabbits and rats dosed with 1-bromo derivatives of butane, pentane, hexane, heptane and octane show that a sulphur-containing metabolite different from the mercapturic acid is present. These unidentified compounds give a colour with sodium nitroprusside and account, at least in part, for the higher values obtained from urines by Barnes *et al.* (1959). The R_F values of these metabolites form a straight line when plotted against the number of carbon atoms in the alkyl radical of the compound fed (see Fig. 1) as do those of the corresponding mercapturic acids and alkylcysteines, suggesting that these metabolites form a homologous series. The decrease in the amount of mercapturic acid formed as the 1-bromo- or 1-iodo-alkane series is ascended might be a consequence of the rate of the acetylation of the alkylcysteine since the percentage of the dose excreted as mercapturic acids by rabbits and rats dosed with the alkylcysteines also decreases as the series is ascended (unpublished work).

The present investigation suggests that the guinea pig is unable to synthesize mercapturic acids to an appreciable extent from any of the halogenoalkanes examined. This is in agreement with the results of Bray *et al.* (1959) which showed that this animal forms either no mercapturic acid or relatively little as compared with rabbit and rat from any type of mercapturic acid precursor.

In all three species the excretion of iodide after the administration of iodoalkanes was of similar magnitude to that observed after dosing with an equimolecular amount of sodium iodide. This indicates that the metabolism of most of the dose involves loss of the iodine atom. In the rabbit and rat we have so far been able to account for only a small percentage of the alkyl radical as mercapturic acid. Another sulphur-containing metabolite is excreted but this probably accounts for about 10% of the dose of 1-bromobutane in the rabbit and rat but much less in the guinea pig, as the colour given by this partially purified metabolite in the nitroprusside reaction is about 2.5 times as intense as that given by the mercapturic acid when the colour intensities are related to their sulphur contents (unpublished work). The excretion of bromide was much slower than that of iodide and incomplete after several days. This is in agreement with the results of previous workers (see Sollmann, 1957). The method of determination used does not distinguish Br^- ions from combined bromine, but as the pattern of excretion resembles that observed after administration of sodium bromide it seems likely that most of a dose of a bromoalkane is debrominated.

SUMMARY

1. The metabolism of some halogeno- and nitro-paraffins has been studied in the rabbit, rat and guinea pig.
2. The corresponding mercapturic acids have been isolated from the urine of rabbits and rats given bromobutane and bromopentane and of rats given bromohexane.
3. The amounts of mercapturic acid excreted by animals given several halogeno- and nitro-alkanes have been determined by a specific chromatographic method.
4. Most of the compounds examined yield in the rabbit and rat another sulphur-containing metabolite, and R_M values suggest that these metabolites from bromobutane to bromo-octane form a homologous series.
5. The excretion of bromide and iodide by animals given bromo- and iodo-alkanes has been measured.

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