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Biochemical Studies of Toxic Agents

14. THE BIOSYNTHESIS OF ETHYLMERCAPTURIC ACID*

BY A. E. R. THOMSON,† E. A. BARNSLEY AND L. YOUNG
Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

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The first evidence for the metabolic formation of an alkylmercapturic acid was obtained by Roberts & Warwick (1957, 1958), who showed by radiochromatographic methods that the administration of ^{14}C -labelled ethyl methanesulphonate,

to rats is followed by the excretion of ethylmercapturic acid, *N*-acetyl-*S*-ethyl-L-cysteine. By the use of a procedure for determining mercapturic acids Bray & James (1958) were led to conclude that the administration of 1- and 2-bromobutane and certain higher 1-bromoalkanes to animals is followed by the excretion of the corresponding alkylmercapturic acids, and Thomson, Maw & Young (1958) showed by chromatographic and radiochromatographic methods that ethylmercapturic acid is excreted by rats dosed with bromoethane. The present paper contains a more detailed

account of the work on ethylmercapturic acid together with a description of the isolation of this compound from the urine of rats dosed with bromoethane or with *S*-ethyl-L-cysteine. While this work was in progress four other alkylmercapturic acids were isolated from the urine of animals dosed with 1-halogenoalkanes, namely *n*-propylmercapturic acid (Grenby & Young, 1959, 1960) and *n*-butyl-, *n*-pentyl- and *n*-hexylmercapturic acid (Bray & James, 1960; James, 1961).

MATERIALS

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

Preparation of S-ethyl-L-cysteine. Method 1. The procedure adopted closely resembled that described by Grenby & Young (1960) for the preparation of *S*-(*n*-propyl)-L-cysteine. Small leaflets of *S*-ethyl-L-cysteine were obtained in 48% yield from 12 g. of L-cystine. After being recrystallized twice from 85% (v/v) methanol in water the compound had m.p. 252–253° (decomp.), $[\alpha]_D^{20} - 27^\circ$ (c 1 in water) (Found: C, 40.5; H, 7.3; N, 9.2; S, 21.6. Calc. for $\text{C}_6\text{H}_{11}\text{NO}_2\text{S}$: C, 40.2; H, 7.4; N, 9.4; S, 21.5%).

* Part 13: Hopkins, Brooks & Young (1962).

† Present address: Division of Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C. 2.

Method 2. This method was similar to that used by Stoll & Seebeck (1948) and by Roberts & Warwick (1958), namely the interaction of iodoethane with L-cysteine in an aq. ethanolic NaOH soln. The crude product was purified by crystallization from aq. 85% (v/v) methanol. The product gave a faint test for cystine with cyanide and nitroprusside and was obtained in a yield of 26%. It was recrystallized from aq. 85% (v/v) methanol and gave a product free from cystine: m.p. 254° (decomp.), $[\alpha]_D^{20} - 25^\circ$ (c 1 in water) (Found: C, 40.0; H, 7.9; N, 9.4; S, 21.8%).

Preparation of N-acetyl-S-ethyl-DL-cysteine. The preparation of N-acetyl-S-ethylcysteine has been described by Roberts & Warwick (1958). Their method, in our hands, yielded an optically inactive product, N-acetyl-S-ethyl-DL-cysteine, m.p. 118–119° (Found: C, 44.2; H, 7.1; N, 7.1; S, 16.5. Calc. for $C_7H_{13}NO_2S$: C, 44.0; H, 6.9; N, 7.3; S, 16.8%). It showed the same R_F values as N-acetyl-S-ethyl-L-cysteine.

Preparation of N-acetyl-S-ethyl-L-cysteine (ethylmercapturic acid). Method 1. S-Ethyl-L-cysteine (1 g.) was dissolved in 28 ml. of ice-cold N-NaOH and 1.75 ml. of acetic anhydride was added drop by drop with vigorous stirring and continuous cooling in an ice bath. More ice-cold N-NaOH (14 ml.) was added, followed by 1.75 ml. of acetic anhydride. After the mixture had been stirred for 5 min. all the droplets of the anhydride disappeared and the solution was brought to pH 1 by the addition of conc. H_2SO_4 . The solution was left overnight at 5° and was then shaken three times with portions (3 vol.) of chloroform. The combined chloroform extracts were evaporated to dryness, the residue was dissolved in 2 ml. of ethanol and 25 ml. of warm carbon tetrachloride was added. The solution was filtered and carbon tetrachloride was added to the filtrate with warming until the volume was 100 ml. The material that separated on cooling was recrystallized from ethanol and carbon tetrachloride and 0.892 g. of crystals was obtained, corresponding to 70% of the theoretical yield: m.p. 78.5–79.5°, $[\alpha]_D^{15} - 21^\circ$ (c 1 in water) (Found: C, 43.7; H, 6.8; N, 7.8; S, 16.8%; equiv. 192. $C_7H_{13}NO_2S$ requires C, 44.0; H, 6.9; N, 7.3; S, 16.8%; equiv. 191).

Method 2. S-Ethyl-L-cysteine (1 g.) prepared by method 1 was acetylated by the action of keten by a procedure similar to that described by Grenby & Young (1960). The gummy product was crystallized from carbon tetrachloride and ethanol; yield, 0.458 g., m.p. 79.0–80.5°, $[\alpha]_D^{18} - 25^\circ$ (c 1 in water) (Found: C, 44.0; H, 6.7; N, 7.4; S, 17.1%; equiv. 193). When 1 g. of S-ethyl-L-cysteine prepared by method 2 was acetylated with keten it yielded 0.872 g. of ethylmercapturic acid, m.p. 78.0–78.5°, not depressed by admixture with the product just described, $[\alpha]_D^{18} - 25^\circ$ (c 1 in water).

Method 3. N-Acetyl-L-cysteine, m.p. 109–110°, $[\alpha]_D^{17} + 7^\circ$ (c 2.7 in water), prepared by the method of Pirie & Hele (1933), was converted into N-acetyl-S-ethyl-L-cysteine by ethylation with iodoethane in liquid ammonia by a procedure closely resembling that described for the preparation of S-ethyl-L-cysteine by method 1. The product was extracted from the reaction mixture with chloroform and crystallized as already described. The yield from 2.5 g. of N-acetyl-L-cysteine was 1.142 g., m.p. 79.0–80.5°, unchanged by admixture with ethylmercapturic acid prepared by method 2, $[\alpha]_D^{18} - 26^\circ$ (c 1 in water) (Found: C, 44.1; H, 6.6; N, 7.5; S, 17.0%; equiv. 192).

Properties of ethylmercapturic acid. The compound was

very soluble in water and in many organic solvents, e.g. methanol, ethanol, acetone, chloroform, dioxan. It was moderately soluble in ether and in benzene, and only slightly soluble in carbon tetrachloride.

When a solution of ethylmercapturic acid in N-HCl was boiled for 2 min. some breakdown to S-ethyl-L-cysteine occurred, as shown by a faint positive ninhydrin reaction. Complete hydrolysis took place when a solution of 0.100 g. of ethylmercapturic acid in 5 ml. of 6N-HCl was refluxed for 3 hr. and evaporated to dryness under reduced pressure. The residue was dissolved in water and evaporated to dryness three times to remove HCl. The residue was neutralized with aq. N-NH₃ soln. and the solution treated with 5 ml. of aq. 85% (v/v) methanol and filtered. After being left at 5° the filtrate gave crystals of S-ethyl-L-cysteine; these were separated, washed with cold methanol and dried. The product (0.037 g.) was free of chloride, and had m.p. 238–240° (decomp.) $[\alpha]_D^{13} - 27^\circ$ (c 1 in water) (Found: C, 40.3; H, 7.5; S, 21.0%). When 5 mg. of ethylmercapturic acid in 2N-NaOH was heated in a boiling-water bath for 15 min. and then acidified, a pungent odour characteristic of ethanethiol was produced. If 3 drops of 4% sodium nitroprusside solution were added instead of acid, a purple colour appeared.

Determination of ethylmercapturic acid. The determination of methionine by reaction with sodium nitroprusside in alkaline solution followed by acidification and the production of a red colour was described by McCarthy & Sullivan (1941). The formation of a red complex with S-ethyl-L-cysteine under these conditions was observed by Thomson (1960). The method of McCarthy & Sullivan (1941) as modified by Maw (1956) has been applied to the determination of ethylmercapturic acid. Measured portions of 1.80 mM-dicyclohexylammonium ethylmercapturate in water were diluted to 5.0 ml., treated with 1.0 ml. of 5N-NaOH and 0.5 ml. of sodium nitroprusside soln. (1.34%, w/v) and, after 15 min. at room temperature, were acidified with 2.0 ml. of 88% (w/w) H_3PO_4 . During the acidification the solution was cooled in ice-water. After 15 min. the extinction was measured at 518 m μ , at which wavelength light-absorption was at a maximum and remained constant for at least 2 hr. Provided that a blank measurement for nitroprusside was deducted, E_{518} varied linearly with the concentration of ethylmercapturic acid, e.g. solutions with final concentrations of dicyclohexylammonium ethylmercapturate of 0.212, 0.424, 0.636, 0.848 and 1.060 mM had respectively these corrected extinctions: 0.115, 0.237, 0.353, 0.476 and 0.597. The method may be applied similarly to solutions of the free acid and to buffered solutions. The reaction was used qualitatively to detect ethylmercapturic acid as it was eluted from ion-exchange resins.

Preparation of the dicyclohexylammonium salt of N-acetyl-S-ethyl-L-cysteine. Well-defined crystalline products were obtained when ethylmercapturic acid reacted with equimolar amounts of certain organic bases, e.g. p-toluidine and benzidine (Thomson, 1960) and dicyclohexylamine. The dicyclohexylammonium salt was prepared by allowing 1.002 g. of ethylmercapturic acid and 0.997 g. of dicyclohexylamine to react in acetone solution. The precipitate that formed was crystallized from 7% (v/v) ethanol in acetone and yielded 1.557 g. of product: m.p. 171–172°, $[\alpha]_D^{17} - 7^\circ$ (c 1.45 in water) (Found: C, 61.1; H, 9.7; N, 7.9; S, 9.0%; equiv. 378. $C_{18}H_{36}N_2O_2S$ requires C, 61.3; H, 9.7;

N, 7.5; S, 8.6%; equiv. 372). The equivalent weight of the dicyclohexylammonium salt was determined by washing samples (4 mg.) with 25 ml. of water through a column (7 cm. $\times$ 1 cm. diam.) of Zeo-Karb 225 resin (50–100 mesh; H^+ form). The liberated acid was titrated in an atmosphere of CO_2 -free N_2 with 0.01 N-NaOH, with phenolphthalein as indicator. A blank titration was carried out on 25 ml. of water passed through the column.

Preparation of ^{35}S -labelled yeast and L- ^{35}S -cystine. A dried-yeast preparation in which the sulphur-containing compounds were labelled with radioactive sulphur (^{35}S -labelled yeast) was obtained by the method of Knight & Young (1958), which was based on that developed by Williams & Dawson (1952).

L- ^{35}S -cystine was prepared from ^{35}S -labelled yeast by the method of Williams & Dawson (1952) as modified by Marsden & Young (1958), and its purity was checked by the constant-solubility test of Gutmann & Wood (1949).

Preparation of S-ethyl-L- ^{35}S -cysteine and N-acetyl-S-ethyl-L- ^{35}S -cystine. S-Ethyl-L- ^{35}S -cysteine was prepared by method 1 described for the non-radioactive compound. The specific activity of the L- ^{35}S -cystine used in the preparation was 122.5 μ C/m-mole, and that of the S-ethyl-L- ^{35}S -cysteine obtained from it was 60.0 μ C/m-mole. Some of this material was converted into ethylmercapturic acid by method 2 and yielded a product with a specific activity of 61.1 μ C/m-mole.

METHODS

Animals and dosing. Male rats, rabbits, guinea pigs and mice were housed in metabolism cages so constructed that the urine was collected separately from the faeces. Urine was collected daily and stored in the refrigerator until used. Except when the diet was modified by the inclusion of radioactive components, the rats, rabbits and mice were given rat cakes [J. Murray and Sons (London) Ltd.] and the guinea pigs were given bran. In addition the rabbits and guinea pigs received cabbage. The animals had access to water at all times.

When ^{35}S -labelled yeast was fed to the animals it was added, to the extent of 5% (w/w), to a diet (Maw, 1953) with the following composition (% by wt.): casein 10, plain flour 25, starch 34.8, margarine 22, dried yeast 4, cod-liver oil 2, Marmite 2, choline 0.2.

Bromoethane (British Drug Houses Ltd.) was administered to the animals as a solution in arachis oil by subcutaneous injection in the lumbar region. Control animals were dosed with arachis oil by the same route. All animals, other than rabbits, were anaesthetized lightly with ether while being injected. Rats (body wt. 150–200 g.), rabbits (body wt. about 3 kg.) and guinea pigs (body wt. about 500 g.) each received bromoethane as a 40% (w/v) solution in arachis oil in amounts ranging from 1.0 to 1.6 g./kg. body wt. for the rats and at 1.0 g./kg. for the rabbits and guinea pigs. The mice (body wt. 18–22 g.) were injected with 1.25 g. of bromoethane/kg. as a 25% solution in arachis oil.

S-Ethyl-L-cysteine was administered to rats (body wt. 150–200 g.) as a 10% (w/v) solution in M-NaHCO₃, or as the hydrochloride in 20% (w/v) solution in water. The NaHCO₃ soln. was administered by subcutaneous or intraperitoneal injection, or by stomach tube, and the solution of the hydrochloride was given by subcutaneous injection.

Paper chromatography. Paper chromatograms were

developed on Whatman no. 1 paper by the ascending or descending method at room temperature. The solvent mixtures used were: A, butan-1-ol-acetic acid-water (4:1:5, by vol.); B, molten phenol-water (4:1, v/v); C, lutidine-collidine-water (6:5:5, by vol.); D, butan-1-ol-pyridine-3N-NH₃ soln. (4:3:3, by vol.); E, 2-methylbutan-2-ol-pyridine-water (7:7:6, by vol.).

Both S-ethyl-L-cysteine and ethylmercapturic acid were detected by spraying with K₂Cr₂O₇-AgNO₃ reagent (Knight & Young, 1958) and dipping in the platinum reagent (Toennies & Kolb, 1951). Ninhydrin, which was used as described by Toennies & Kolb (1951), was employed for the detection of S-ethyl-L-cysteine.

Radiochromatography. Paper chromatograms were prepared by the methods already described and radioactive compounds were located either by section scanning or by automatic scanning. Section scanning was carried out by the method described by Lowenstein (1953; see also Corner & Young, 1955). Section scanning is time-consuming and this procedure was replaced by automatic scanning, with the equipment and procedure developed by Morrison & Young (1959).

Measurement of ^{35}S . Compounds containing radioactive sulphur were oxidized by the Carius procedure and ^{35}S was counted as benzidine sulphate as described by Hawkins & Young (1954) and Marsden & Young (1958).

RESULTS

Metabolic formation of ethylmercapturic acid from bromoethane

Paper chromatography. Bromoethane was administered by subcutaneous injection to rats, rabbits, guinea pigs and mice, and the urine was collected for the first 24 hr. period after dosing. Each specimen of urine was filtered through cotton wool or was centrifuged and was then examined by paper chromatography in three solvent mixtures before acid hydrolysis, and in two solvent mixtures after it had been boiled under reflux with an equal volume of concentrated hydrochloric acid for 90 min. A sample of the urine was also acidified to pH 1–2 with concentrated hydrochloric acid and extracted three times with 3 vol. of chloroform. After each extraction the mixture was centrifuged and the clear chloroform extract was passed through a dry filter paper. Part of the combined chloroform extracts was concentrated to a small volume and examined by chromatography in three solvent mixtures. The remainder was evaporated to dryness, the residue was boiled under reflux for 90 min. with 6 N-hydrochloric acid and was then chromatographed. Results obtained with rats are shown in Table 1.

The urine of rats dosed with bromoethane, and chloroform extracts of this urine, gave spots with potassium dichromate-silver nitrate reagent corresponding to those obtained with ethylmercapturic acid. No comparable spots were obtained with the control urine from rats dosed with arachis oil, or

with chloroform extracts of this urine. When the urine of the dosed rats and the chloroform-soluble fraction from the acidified urine were hydrolysed by refluxing with hydrochloric acid, the chromatograms no longer showed a spot corresponding to ethylmercapturic acid, but instead showed a new spot corresponding to *S*-ethyl-L-cysteine. This compound was revealed by its reactions with both the potassium dichromate-silver nitrate reagent and the ninhydrin reagent. Similar results were obtained when control urine to which ethylmercapturic acid had been added was hydrolysed with hydrochloric acid and then examined by paper chromatography. After hydrolysis with hydrochloric acid *S*-ethyl-L-cysteine could be detected neither in the control urine nor in the chloroform-soluble fraction of the acidified control urine. Ethylmercapturic acid could not be detected in urine excreted by rats in the second 24 hr. period after they had been dosed with bromoethane. Although the absence of *S*-ethyl-L-cysteine from the acid-hydrolysed urine of animals dosed with bromoethane would have indicated that the animals did not excrete ethylmercapturic acid, the *S*-ethyl-L-cysteine found in the acid-hydrolysed urine may have been derived in part from ethylmercapturic acid sulphoxide (Thomson & Young, 1960).

Experiments similar to those just described were also conducted with the urine of rabbits, guinea pigs and mice that had been injected subcutaneously with arachis oil or with a solution of bromoethane in arachis oil. Results were substantially the same as those shown in Table 1 for experiments with rats. The excretion of ethylmercapturic acid by guinea pigs dosed with bromoethane was insufficient to permit its detection in the urine itself, although it was detected in chloroform extracts of the acidified urine. By paper chromatography it was possible to show the presence of ethylmercapturic acid in the urine, and in chloroform extracts of the acidified urine, of rats that had been injected subcutaneously with 0.5 g. of iodoethane/kg. body wt.

Radiochromatographic studies. Four male rats, each weighing about 170 g., were housed in

separate metabolism cages and were transferred to clean cages at the end of each day of the experiment. Each rat was provided daily during the first 3 days with 20 g. of the food mixture containing 1 g. of ^{35}S -labelled yeast with specific activity 0.3 $\mu\text{C}/\text{mg}$. (cf. Knight & Young, 1958). At the end of 3 days, two rats were each given a subcutaneous injection of 0.25 g. of bromoethane as a 40% solution in arachis oil, and the other two rats were each injected with the same volume of arachis oil alone. After being dosed the animals were given the same diet as before except that ^{35}S -labelled yeast was not included. Three consecutive 24 hr. samples of urine were collected. Samples of urine excreted in the first 24 hr. period after the injections were submitted to paper chromatography before and after acid hydrolysis, as also were the chloroform-soluble fractions from the acidified urine. The procedures followed were essentially those already described for non-radioactive experiments, except that in addition to being sprayed with detecting agents the chromatograms were also scanned for ^{35}S .

Table 2 shows that with all three solvent mixtures the urine, and the chloroform-soluble fraction of the urine, of the dosed animals gave rise to radioactive spots with R_F values corresponding to ethylmercapturic acid. These radioactive spots gave positive tests with potassium dichromate-silver nitrate reagent and with the platinum reagent. Moreover, when the urine and the chloroform-soluble fraction of the urine were hydrolysed with hydrochloric acid they yielded chromatograms with radioactive spots with R_F values corresponding to those of *S*-ethyl-L-cysteine. These spots gave positive reactions with ninhydrin and with potassium dichromate-silver nitrate reagent. No corresponding spots were found on the radiochromatograms of the control urines. Furthermore, ethylmercapturic acid could not be detected by radiochromatographic examination of the urine excreted by rats in the second and third 24 hr. periods after they had been dosed with bromoethane.

Experiments similar to those just described have provided evidence that mice also excrete ethyl-

Table 1. *Chromatographic examination of urine excreted by rats in the first 24 hr. period after subcutaneous injection of bromoethane*

						For details see text.					
						R_F					
Solvent mixture	...	...	...	...	...	A	B	C	A	B	E
Ethylmercapturic acid						0.85	0.78	0.55	—	—	—
<i>S</i> -Ethyl-L-cysteine						—	—	—	0.44	0.73	0.55
						Before acid hydrolysis			After acid hydrolysis		
Urine						0.84	0.81	0.59	0.47	0.73	—
Chloroform-soluble fraction of acidified urine						0.86	0.78	0.53	0.45	—	0.53

mercapturic acid in the first 24 hr. period after they have been injected subcutaneously with bromoethane.

In addition to the spots corresponding to ethylmercapturic acid in the radiochromatographic experiments with the urine of rats dosed with bromoethane, a second radioactive spot was found with R_f 0.47 in solvent mixture A and R_f 0.28 in solvent mixture D. In solvent mixture B the radioactivity of the spot corresponding to ethylmercapturic acid was such as to suggest that the second ^{35}S -labelled compound was running with the same R_f as ethylmercapturic acid. The second radioactive compound gave no reaction with potassium dichromate-silver nitrate reagent or with ninhydrin. After the urine had been refluxed with hydrochloric acid neither ethylmercapturic acid nor the second compound was present on the radiochromatogram, but instead there was a single radioactive spot that gave positive reactions with potassium dichromate-silver nitrate reagent and with ninhydrin and corresponded in R_f to *S*-ethyl-L-cysteine. As the control urine did not give these reactions it appears that bromoethane gave rise to the excretion of a sulphur-containing compound in addition to ethylmercapturic acid. Thomson & Young (1960) found that the compound was the sulphoxide of ethylmercapturic acid.

Isolation of ethylmercapturic acid. A group of 25 male rats were each injected subcutaneously with 0.5 ml. of a 40% (w/v) solution of bromoethane in arachis oil and the urine was collected for the following 24 hr. Three days later the dosing with bromoethane was repeated and the urine was again collected for the next 24 hr. The total urine (710 ml., pH 7) was concentrated to 170 ml. at 40–45° under reduced pressure and then centrifuged. The supernatant liquid was brought to

pH 1–2 with concentrated hydrochloric acid and extracted six times by shaking with 300 ml. portions of chloroform. A seventh chloroform extract, after it had been evaporated to dryness and then dissolved in water, gave no test for ethylmercapturic acid by the nitroprusside reaction. The combined chloroform extracts were evaporated to dryness under reduced pressure and neutralized by addition of *N*-sodium hydroxide. This solution was diluted to 100 ml. with water and fractionated on a resin column. Amberlite CG-400 (100–200 mesh; Cl^- form) was made into a slurry with water and packed into a column 36 cm. $\times$ 2 cm. The column was washed with *M*-sodium formate solution until free from chloride and it was then equilibrated with a sodium formate buffer, pH 4.1, which was 0.1 *M* with respect to its total formate content. A disk of filter paper was placed on the resin to stabilize the surface, and the solution of material extracted from the urine was transferred to the column. The walls of the column were washed down with a few millilitres of 0.1 *M*-formate buffer, and elution was carried out by a concentration-gradient technique in which the outflow of eluent from a flask originally containing 700 ml. of 0.1 *M*-formate buffer, pH 4.1, was compensated by an inflow of 0.3 *M*-formate buffer, pH 4.1. The concentration gradient was approximately linear over the eluate volume collected. The flow rate was 2 ml./min., and 25 ml. fractions were collected.

Fractions 17–37 inclusive gave a positive response to the nitroprusside test with maximum reaction in fraction 27. Fractions 17–37 were combined, diluted to 1 l. and passed down a column, 45 cm. $\times$ 2 cm., of Zeo-Karb 225 (50–100 mesh; H^+ form) followed by 200 ml. of water. The eluate was evaporated to dryness under

Table 2. *Radiochromatographic examination of urine excreted by rats in the first 24 hr. period after they had received a diet containing ^{35}S -labelled yeast for 3 days and had then been injected subcutaneously with bromoethane*

For details see text. The R_f values reported for ethylmercapturic acid and *S*-ethyl-L-cysteine were obtained by chemical tests; all other R_f values are for peaks on the radiochromatograms.

Solvent mixture	R_f					
	A	B	C	A	B	D
Ethylmercapturic acid	0.78	0.82	0.50	—	—	—
<i>S</i> -Ethyl-L-cysteine	—	—	—	0.45	0.76	0.42
Urine	Before acid hydrolysis			After acid hydrolysis		
	0.77*†	0.82*†	0.46*†	0.46‡	0.75‡	0.41‡
Chloroform-soluble fraction of acidified urine	0.47†	—	0.28†	—	—	—
	0.80*†	0.75*†	0.52*†	0.46‡	0.77‡	0.43‡
	0.50†	—	0.30†	—	—	—

* Positive reaction with the $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 reagent of Knight & Young (1958).

† Positive reaction with the platinum reagent of Toennies & Kolb (1951).

‡ Positive reaction with both the above reagents and with ninhydrin reagent.

reduced pressure and the residue was brought to pH 5 with 0.5N-sodium hydroxide. This solution was diluted to 10 ml. and the fractionation was repeated on a fresh column of Amberlite CG-400. The ethylmercapturic acid appeared in fractions 20-31 inclusive. These were combined and passed through a column of Zeo-Karb 225 as before. The eluate was evaporated to dryness under reduced pressure and the pale-yellow gum was dissolved in 20 ml. of water and passed through a column of Zeo-Karb 225, 10 cm. $\times$ 1 cm., to remove the last traces of cations. The resin was washed with water until the eluate was pH 4-5, and the total eluate was then evaporated to dryness under reduced pressure. The residue was dried over phosphorus pentoxide and solid sodium hydroxide *in vacuo*. The gummy product thus obtained was dissolved in 10 ml. of ethanol-acetone (1:1, v/v) and a 30% (v/v) solution of dicyclohexylamine in acetone was added until a drop of the solution evaporated on indicator paper gave pH 6-7. The solution was evaporated in a current of air and the residue was then dried over phosphorus pentoxide *in vacuo*. The white crystalline solid, contaminated with some yellow gum, was stirred with 4 ml. of ethanol. Some insoluble material was removed by centrifuging and the supernatant solution was evaporated to dryness in an air current and dried over phosphorus pentoxide. The residue was dissolved in the minimum volume of warm ethanol and 8 ml. of warm acetone was added. Crystallization was allowed to proceed for 12 hr. at 5° and the product was separated, washed with a little acetone and then recrystallized. The dicyclohexylammonium salt of biosynthetic ethylmercapturic acid thus obtained weighed 0.170 g., which corresponds to 0.5% of the administered bromoethane; m.p. 170.5-171.5°, mixed m.p. with an authentic sample, 170-171°, $[\alpha]_D^{25} - 7^\circ$ (c 1.7 in water) (Found: C, 61.5; H, 9.7; N, 7.2; S, 8.5%; equiv. 384. Calc. for $C_{19}H_{36}N_2O_3S$: C, 61.3; H, 9.7; N, 7.5; S, 8.6%; equiv. 372). The compound gave 99.4% of the theoretical value when assayed for ethylmercapturic acid by the nitroprusside method. When examined by paper chromatography in solvent mixtures A and D it showed R_F 0.82 and 0.50 respectively, whereas values obtained with the authentic compound were R_F 0.84 and 0.51. The detecting agents used were potassium dichromate-silver nitrate reagent and platinum reagent. When the biosynthetic compound was hydrolysed by heating in a sealed tube at 95° with 6N-hydrochloric acid, it was converted into *S*-ethyl-L-cysteine, which had R_F 0.47 and 0.45 in solvent mixtures A and D respectively. The R_F values of authentic *S*-ethyl-L-cysteine in these solvent mixtures were 0.46 and 0.46. Detection of the compound was with the platinum reagent and with ninhydrin.

Metabolic formation of ethylmercapturic acid from S-ethyl-L-cysteine

Paper chromatography. Four rats were each dosed subcutaneously with 0.30 g. of *S*-ethyl-L-cysteine hydrochloride as a 20% (w/v) solution in water. The urine was collected for 24 hr. after dosing and was examined for the presence of ethylmercapturic acid. The results show that the urine contained a compound with the same R_F values as ethylmercapturic acid, and that after acid hydrolysis this compound was replaced by one with R_F values corresponding to *S*-ethyl-L-cysteine (Table 3).

Radiochromatography. One male rat (body wt. 190 g.) was injected subcutaneously with 80 mg. of *S*-ethyl-L- ^{35}S -cysteine in 1.5 ml. of M-sodium hydrogen carbonate, and the urine excreted in each of the three ensuing 24 hr. periods was collected. The specific activity of the administered compound was 60 μC /m-mole. When the urine excreted in the first 24 hr. period was run in solvent mixture D it showed a radioactive spot with R_F 0.53 corresponding to 29% of the ^{35}S administered. This spot gave a positive reaction with the platinum reagent and no reaction with ninhydrin. A sample of ^{35}S -ethylmercapturic acid run alongside behaved in exactly the same manner. When the urine was hydrolysed by heating 0.5 ml. with 0.5 ml. of concentrated hydrochloric acid in a sealed tube for 4 hr. at 90° and was then chromatographed in solvent mixture D, no evidence for the continued presence of ethylmercapturic acid was obtained. The chromatogram showed instead a radioactive spot that gave a positive reaction with the platinum reagent and with ninhydrin and had the same R_F , 0.36, as *S*-ethyl-L- ^{35}S -cysteine run alongside. Ethylmercapturic acid could not be detected in the urine excreted during the second and third 24 hr. periods after dosing with *S*-ethyl-L- ^{35}S -cysteine. In similar experiments rats dosed with *S*-ethyl-L- ^{35}S -cysteine by stomach tube and by intraperitoneal injection excreted ethyl ^{35}S mercapturic acid.

Table 3. *Chromatographic examination of urine excreted by rats in the first 24 hr. period after subcutaneous injection of S-ethyl-L-cysteine*

For details see text.			R_F		
Solvent mixture	...	...	A	B	D
Ethylmercapturic acid			0.87	0.83	0.56
<i>S</i> -Ethyl-L-cysteine			0.53	0.77	0.46
Before acid hydrolysis					
Urine			0.86	0.81	0.53
Chloroform-soluble fraction of acidified urine			0.87	0.81	0.54
After acid hydrolysis					
Urine			0.53	0.77	0.47

Isolation of ethylmercapturic acid. Four male rats were each dosed by subcutaneous injection with 0.075 g. of *S*-ethyl-L-cysteine in 1.5 ml. of *m*-sodium hydrogen carbonate. The urine (pH 8; total volume, 45 ml.) was collected for the first 24 hr. Part of the urine (27 ml.) was diluted to 100 ml., transferred to a column (38 cm. $\times$ 3 cm.) of Amberlite CG-400 (100–200 mesh; formate form) equilibrated at pH 4.1 and subjected to gradient elution. The mixing vessel contained 1.8 l. of formate buffer, pH 4.1, initially 0.1 M with respect to its content of total formate. This yielded 0.121 g. of the dicyclohexylammonium salt of ethylmercapturic acid: m.p. 170.5–172°, unchanged by admixture with the authentic compound, $[\alpha]_D^{25} - 7^\circ$ (c 1.5 in water) (Found: C, 61.5; H, 10.0; N, 7.5; S, 8.3 %). Assay for ethylmercapturic acid by the reaction with nitroprusside gave the theoretical value. The amount of product isolated corresponded to 27 % of the administered *S*-ethyl-L-cysteine. When the experiment was repeated with another group of four rats, a conversion of 20 % was obtained.

DISCUSSION

The fact that alkyl halides such as bromomethane, bromoethane and iodoethane are broken down in the animal body with the liberation of halogen ions was demonstrated by Blumgart, Gilligan & Swartz (1931) and by Miller & Haggard (1943), but was attributed to the occurrence of hydrolytic reactions. Though it is to be expected that some hydrolysis of an alkyl halide will occur *in vivo*, it is now apparent that it may undergo other types of reaction, one of which is the formation of an alkylmercapturic acid.

The amounts of ethylmercapturic acid isolated

in the present work from the urine of rats dosed with bromoethane corresponded to less than 1 % of the bromoethane administered. Bromoethane (b.p. 38°) is very volatile, however, and Miller & Haggard (1943) observed that when it was injected into rats 73–89 % was eliminated unchanged in the expired air. If allowance is made for this fact and also for the losses that occur during the isolation of ethylmercapturic acid from urine, it is apparent that in a quantitative sense this compound is a more important metabolite of bromoethane than the amounts isolated would suggest. Moreover, the metabolic conversion of ethylmercapturic acid into derivatives such as ethylmercapturic acid sulphoxide (Thomson & Young, 1960) must also be taken into account. When bromoethane is administered to rats that have received ^{35}S -labelled yeast in their diet, it has almost no effect on the excretion of ^{35}S in the total-sulphate fraction of the urine, but brings about a marked increase in the excretion of neutral-sulphur ^{35}S (Thomson, 1960), and further work may reveal that this fraction contains additional metabolites derived from bromoethane. Bray & James (1960) and James (1961) have reported that the urine of rabbits dosed with 1-bromobutane contains, in addition to butylmercapturic acid, two other sulphur-containing metabolites, one of which gives *S*-butyl-L-cysteine and glycine on hydrolysis.

In recent years considerable attention has been paid to the mechanism of formation of mercapturic acids. Barnes, James & Wood (1959) have shown that the concentration of glutathione in the liver of rats falls when mercapturic acid precursors, including 1-bromobutane, are administered, and that the fall corresponds approximately to the amount of mercapturic acid formed. These findings,

Fig. 1. Biosynthesis of ethylmercapturic acid. Conversions demonstrated in the animal body are shown by solid arrows and a possible metabolic pathway is indicated by broken arrows.

together with later work, led Bray, Franklin & James (1959) to conclude that their results were 'compatible with the view that stages in the formation of mercapturic acids *in vivo* are the combination of the precursor with glutathione in the liver and subsequent hydrolysis of the glutathione complex by glutathionase to *S*-substituted cysteine, glycine and glutamic acid'. This conclusion of Bray *et al.* (1959) has been supported by the findings of E. Boyland and his co-workers. Booth, Boyland & Sims (1960) demonstrated that rat-liver slices convert naphthalene into a compound that they identified as *S*-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione. Further, Boyland, Ramsay & Sims (1961) showed that this glutathione derivative is present in the bile of rats that have been dosed with naphthalene, and that the bile also contains the corresponding *S*-cysteinylglycine, *S*-cysteine and *N*-acetyl-*S*-cysteine conjugates. Booth, Boyland & Sims (1961) have also shown that bromoethane reacts with reduced glutathione in the presence of fresh and of boiled soluble liver fraction, although the reaction is faster in the presence of the former. In view of these observations the possibility arises that glutathione participates in the formation of ethylmercapturic acid. In the present work ethylmercapturic acid has been isolated from the urine of rats dosed with bromoethane and with *S*-ethyl-L-cysteine, and Roberts & Warwick (1958), by means of radiochromatographic procedures, found that the same acid is formed when rats are injected with *S*-ethylglutathione. As shown in Fig. 1, it thus becomes possible to visualize bromoethane as giving rise to ethylmercapturic acid by reaction with glutathione to form *S*-ethylglutathione (I), which is broken down to *S*-ethyl-L-cysteine (II); this then undergoes acetylation to give ethylmercapturic acid (III). It is possible, however, that bromoethane brings about the direct ethylation of cysteine and cysteine-containing compounds such as proteins, and that, even if glutathione does participate in the formation of ethylmercapturic acid, there may be other pathways for the biosynthesis of this alkylmercapturic acid.

SUMMARY

1. *N*-Acetyl-*S*-ethyl-L-cysteine (ethylmercapturic acid) has been prepared by three methods and some of its properties have been reported.

2. The excretion of ethylmercapturic acid by rats, rabbits, guinea pigs and mice after they have been injected subcutaneously with bromoethane has been demonstrated by paper-chromatographic examination of the urine. In rats and mice this finding has been confirmed by radiochromatographic methods.

3. Ethylmercapturic acid has been isolated in

the form of its dicyclohexylammonium salt from the urine of rats dosed with bromoethane by subcutaneous injection.

4. *S*-Ethyl-L-cysteine has been shown by chromatographic and radiochromatographic methods to be converted into ethylmercapturic acid in rats, and the mercapturic acid has been isolated from the urine of dosed animals.

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