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Biochemical Studies of Toxic Agents

15. THE BIOSYNTHESIS OF ETHYLMERCAPTURIC ACID SULPHOXIDE*

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The oxidation of a number of thio ethers to *S*-oxides (sulphoxidation) has been observed in biological systems. Compounds metabolized by mammals to the sulphoxide include chlorpromazine (Salzman & Brodie, 1956), 1,2-diphenyl-4-(phenylthioethyl)pyrazolidine-3,5-dione (Burns *et al.* 1957) and promazine (Walkenstein & Seifter, 1959). The oxidation occurs in micro-organisms: (−)-biotin sulphoxide is produced by *Aspergillus niger* (Wright, Cresson, Valiant, Wolf & Folkers, 1954) and 17β-acetoxy-7α-methylthioandrost-4-en-3-one is converted by *Calonectria decora* into 17β-hydroxy-7α-methylsulphinylandrost-4-en-3-one (Holmlund *et al.* 1962). Several examples of the occurrence of sulphoxides in plants are known. They are principally *S*-alkyl-L-cysteine *S*-oxides, their derivatives and ω-methylsulphinylalkyl isothiocyanate glucosides, and they have been reviewed recently by Virtanen (1962).

The sulphoxidation of 4,4′-diaminodiphenyl sulphide has been observed *in vitro* with a preparation of guinea-pig-liver microsomes which required $NADPH_2$ and oxygen for maximum activity (Gillette & Kamm, 1960), and evidence for the enzymic sulphoxidation of *S*-methyl-L-cysteine in leaves has been presented by Arnold & Thompson (1962).

Evidence for the occurrence of ethylmercapturic acid sulphoxide (*N*-acetyl-*S*-ethyl-L-cysteine *S*-oxide) in the urine of rats that had been dosed with bromoethane was presented by Thomson & Young (1960). The present paper gives a fuller account of this work together with evidence for the metabolic conversion of *S*-ethyl-L-cysteine, *S*-ethyl-L-cysteine *S*-oxide and ethylmercapturic acid into ethylmercapturic acid sulphoxide, and describes the isolation of (+)-ethylmercapturic acid sulphoxide, as its dicyclohexylammonium salt, from the urine of rats to which *S*-ethyl-L-cysteine had been administered.

MATERIALS

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

Preparation of S-*ethyl*-L-*cysteine* S-*oxide*. The oxidation of *S*-(*n*-propyl)-L-cysteine to the sulphoxide in aqueous solution with H_2O_2 was described by Stoll & Seebeck (1951), and a similar procedure has been applied to the oxidation of *S*-ethyl-L-cysteine prepared by method 2 of Thomson, Barnsley & Young (1963). To a suspension of 0·697 g. of *S*-ethyl-L-cysteine in 8 ml. of water was added 0·55 ml. of 30% (w/v) H_2O_2. The mixture was shaken occasionally and after 4 hr. all the *S*-ethyl-L-cysteine had dissolved. After 24 hr. at room temperature the solution was evaporated under reduced pressure to give a white residue that was crystallized twice from a mixture of 1·5 ml. of water and 15 ml. of ethanol. The product weighed 0·642 g., m.p. 159–160° (decomp.), $[\alpha]_D^{19}$ −18° (*c* 1 in water) (Found: C, 36·0; H, 6·7; N, 8·5. Calc. for $C_5H_{11}NO_3S$: C, 36·4; H, 6·7; N, 8·5%). This product is considered to be a mixture of two diastereoisomers, since *S*-methyl-L-cysteine *S*-oxide (Synge & Wood, 1956) and *S*-(*n*-propyl)-L-cysteine *S*-oxide (Stoll & Seebeck, 1951), prepared by a similar method, have been resolved.

Preparation of (−)-N-*acetyl*-S-*ethyl*-L-*cysteine* S-*oxide* [(−)-*ethylmercapturic acid sulphoxide*]. Ethylmercapturic acid (26·2 g.), prepared by the method of Thomson *et al.* (1963), was dissolved in 400 ml. of water and oxidized with 16·3 ml. of 30% (w/v) H_2O_2. After 16 hr. at room temperature the solution was evaporated under reduced pressure. The residue was dissolved in ethanol (50 ml.) and crystallized by the addition of ethyl acetate (900 ml.). After the solution had stood overnight at 5° the crude (−)-isomer was filtered off. The residual liquors were retained for the separation of the (+)-isomer as the dicyclohexylammonium salt. Two recrystallizations of the (−)-isomer from a mixture of ethanol (100 ml.) and ethyl acetate (900 ml.) yielded 7·6 g., m.p. 126·5–127° (decomp.), $[\alpha]_D^{21}$ −97° (*c* 1·0 in water) (Found: C, 40·5; H, 6·2; N, 6·8; S, 15·4; equiv. wt. 206. $C_7H_{13}NO_4S$ requires C, 40·6; H, 6·3; N, 6·8; S, 15·5%; equiv. wt. 207). The specific rotation could not be increased by recrystallizing the product from other solvent mixtures.

Preparation of the dicyclohexylammonium salt of (−)-*ethylmercapturic acid sulphoxide*. This was obtained by the method described for the preparation of dicyclohexylammonium ethylmercapturate by Thomson *et al.* (1963). (−)-Ethylmercapturic acid sulphoxide (2·06 g.) was dissolved in 250 ml. of acetone containing a few millilitres of ethanol, and 1·98 g. of dicyclohexylamine was added. Crystallization occurred immediately and the salt was recrystallized from 9·5% (v/v) ethanol in acetone to give 3·14 g. of product, m.p. 167–168° (decomp.), $[\alpha]_D^{17}$ −39° (*c* 1·6 in water) (Found: C, 58·5; H, 9·4; N, 7·1; S, 8·2. $C_{19}H_{36}N_2O_4S$ requires C, 58·7; H, 9·3; N, 7·2; S, 8·2%). Further recrystallization from mixtures of ethanol and acetone or of chloroform and light petroleum (b.p. 40–60°)

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did not increase the specific rotation of the salt, and the acid, when recovered, had $[\alpha]_D^{20} - 95°$ (*c* 1·0 in water), m.p. 126·5–127° (decomp.).

Preparation of the dicyclohexylammonium salt of (+)-ethylmercapturic acid sulphoxide. It was not possible to crystallize (+)-ethylmercapturic acid sulphoxide from the oxidation products of ethylmercapturic acid, but the dicyclohexylammonium salt was crystallized. The residual liquor obtained when (−)-ethylmercapturic acid sulphoxide had been crystallized from a solution of the oxidation products of ethylmercapturic acid was evaporated to a syrup under reduced pressure. This was dissolved in ethanol (10 ml.) and the solution was made just turbid by the addition, at room temperature, of ethyl acetate. After 14 hr. at 5° a gum containing some crystals was separated from the solution. Acetone (200 ml.) was added to the solution followed by a slight excess of dicyclohexylamine over that required to neutralize the acid present, and crystallization was allowed to occur at 5°. The product weighed 9·8 g., $[\alpha]_D^{19} + 14°$ (*c* 1·5 in water). Two recrystallizations from 3% (v/v) chloroform in carbon tetrachloride raised the specific rotation to $[\alpha]_D^{20} + 16°$ (*c* 1·5 in water). The specific rotation was not increased on further recrystallization from the same solvent or from mixtures of chloroform and light petroleum (b.p. 40–60°) or of ethanol and light petroleum (b.p. 40–60°). The product had m.p. 163·5–164·5° (decomp.) (Found: C, 58·5; H, 9·2; N, 7·0; S, 8·3%).

Some properties of ethylmercapturic acid sulphoxide and related compounds. (−)-Ethylmercapturic acid sulphoxide was hygroscopic. It was very soluble in water, methanol, ethanol and chloroform, moderately soluble in acetone and slightly soluble in carbon tetrachloride. When an aqueous solution of the (+)-isomer, obtained by passing a solution of the purified dicyclohexylammonium salt through Zeo-Karb 225 resin (H^+ form), was evaporated, a syrup was obtained that solidified on being dried over P_2O_5 *in vacuo*. This was hygroscopic and could not be crystallized. It had $[\alpha]_D^{15} + 65°$ (*c* 1·2 in water). A comparison of the infrared-absorption spectra of (+)- and (−)-dicyclohexylammonium ethylmercapturate sulphoxide was kindly made by Dr W. O. George who commented as follows: 'As a mull in nujol the dextrorotatory isomer has absorption bands at 1018 and 1050 cm.$^{-1}$ which may be assigned to sulphoxide. The characteristic absorptions of an ammonium or *N*-substituted ammonium salt of an organic acid are present. There is also a band at 3330 cm.$^{-1}$ which could be the secondary amide N–H stretch. The assignment of the amide I carbonyl is anomalous, since two bands appear in this region at 1630 and 1670 cm.$^{-1}$. These could arise from *cis–trans* isomerism, or alternatively the higher band could be assigned to a C=N arising from a tautomeric shift. The laevorotatory isomer differs from the dextrorotatory isomer in having three bands in the sulphoxide region at 1023, 1040 and 1060 cm.$^{-1}$. This could be due to different crystal field effects of the diastereoisomers.'

Sulphoxides are racemized in strong acid solution (Iselin, 1961; Price & Oae, 1962). When 0·907 g. of (+)-dicyclohexylammonium ethylmercapturate sulphoxide was dissolved in 50 ml. of 20 N-formic acid, the specific rotation fell from +50° to +3° over a period of 13 days. (−)-Ethylmercapturic acid sulphoxide (0·174 g.) was recovered from the solution and had $[\alpha]_D^{15} - 92°$ (*c* 1 in water), m.p. 126·5° (decomp.), unchanged on admixture with the authentic isomer. Under the conditions described below for the isolation of biosynthetic ethylmercapturic acid sulphoxide, the strength of the formic acid solution rose during evaporation at 45° to about 16 N; a solution of (+)-dicyclohexylammonium ethylmercapturate sulphoxide in 16 N-formic acid showed no significant decrease in optical rotation after 6 hr. at 45°.

S-Methyl-L-cysteine *S*-oxide gives *S*-methyl-L-cysteine on heating with N-HCl (Morris & Thompson, 1956) and with 6 N-HCl (Synge & Wood, 1956) in yields of 38 and 40–50% respectively. Zahn & Baschang (1959) observed that lanthionine sulphoxide in 6 N-HCl at 105° gives about 25% of lanthionine and they suggested that the reducing agent was HCl. Ostermayer & Tarbell (1960), however, have proposed that the very small amount of *S*-methyl-L-cysteine that they observed when *S*-methyl-L-cysteine *S*-oxide was boiled with N-HCl arose from the reductive removal of sulphoxide oxygen by methanesulphenic acid or by methyl methanethiolsulphinate which are other intermediate products of the reaction. We have observed chromatographically that the decomposition of (−)-ethylmercapturic acid sulphoxide, (+)- and (−)-dicyclohexylammonium ethylmercapturate sulphoxide and *S*-ethyl-L-cysteine *S*-oxide with HCl leads to the formation of *S*-ethyl-L-cysteine. When a solution of 0·60 g. of (−)-ethylmercapturic acid sulphoxide in 10 ml. of 5 N-HCl was refluxed for 3 hr. a product was obtained that, after crystallization from methanol, gave 0·062 g. of *S*-ethyl-L-cysteine, m.p. 247° (decomp.), $[\alpha]_D^{20} - 23°$ (*c* 1·0 in water) (Found: C, 40·5; H, 7·2; N, 9·4; S, 21·2. Calc. for $C_5H_{11}NO_2S$: C, 40·2; H, 7·4; N, 9·4; S, 21·5%).

The reduction of ethylmercapturic acid sulphoxide by thioglycollic acid was reported by Thomson & Young (1960), and Iselin (1961) used thioglycollic acid to reduce methionine sulphoxide to methionine. A solution of (−)-ethylmercapturic acid sulphoxide (0·20 g.) in 40 ml. of water was adjusted to pH 3 and heated under N_2 with 0·67 ml. of thioglycollic acid for 4 hr. at 80–85°. The reaction mixture was cooled to 0°, diluted with 10 ml. of water and treated with 17·5 ml. of a 15% (w/v) solution of $HgCl_2$ in methanol. After filtration, 0·094 g. of product was separated from the filtrate and after recrystallization it had m.p. 76–78° and the same R_F values in solvents A and B (defined below) as an authentic sample of ethylmercapturic acid. A similar reduction of the dicyclohexylammonium salts of both (+)- and (−)-ethylmercapturic acid sulphoxide was demonstrated by chromatography of the reaction mixture with solvent B. When solvent A was used thioglycollic acid or products derived from it ran in the position expected for ethylmercapturic acid.

Preparation of S-*ethyl*-L-*cysteine* SS-*dioxide* (*ethylcysteine sulphone*). *S*-Ethyl-L-cysteine (2·08 g.) was dissolved in 100 ml. of N-H_2SO_4 and oxidized at room temperature with a solution of $KMnO_4$ (2·93 g.) in 200 ml. of water. Manganese dioxide was filtered off and a saturated solution of $Ba(OH)_2$ was added to the filtrate until all the sulphate had been precipitated. The solution, which was now alkaline, was filtered and the filtrate was passed through a column (47 cm. × 2 cm.) of Zeo-Karb 225 resin (50–100 mesh; H^+ form). The column was washed with 800 ml. of water after which amino acids were displaced with 1 l. of N-NH_3 solution. The eluate was evaporated at 40–50° under reduced pressure and gave a white solid (1·40 g.) which was crystallized twice from 33% (v/v) water in ethanol to give 1·09 g. of product, m.p. 175–176° (decomp.), $[\alpha]_D^{16} - 13°$ (*c* 1·2 in

water) (Found: C, 33·0; H, 5·9; N, 7·8; S, 17·8. Calc. for $C_5H_{11}NO_4S$: C, 33·1; H, 6·1; N, 7·7; S, 17·7%). Potentiometric titration of an aqueous solution of the compound indicated the presence of groups ionizing with pK_a values 1·5 and 7·9 (approx.); the alkali titration, which was carried out under CO_2-free N_2, indicated an equivalent weight of 182 (calc. 181). Roberts & Warwick (1958) prepared *S*-ethylcysteine sulphone, m.p. 169°, by oxidizing ethylcysteine with H_2O_2.

Preparation of N-*acetyl*-S-*ethyl*-L-*cysteine* SS-*dioxide* (*ethylmercapturic acid sulphone*). *S*-Ethyl-L-cysteine *SS*-dioxide (0·271 g.) was dissolved in 10 ml. of N-NaOH and acetylated with keten at 15–16°. The solution was kept alkaline to phenolphthalein during the reaction by the addition of 5N-NaOH. When a sample no longer gave a reaction with ninhydrin the solution was diluted to 100 ml. and passed through a column (20 cm. × 2 cm. diam.) of Zeo-Karb 225 resin (50–100 mesh; H^+ form). The column was washed with water until the pH of the eluate rose to 4–5. The total eluate was then evaporated at 40–50° and gave a syrup that crystallized on standing over P_2O_5 and solid NaOH *in vacuo*. The product was recrystallized twice from 10 ml. of warm acetone by the addition of warm benzene (40 ml.); the yield was 0·146 g., m.p. 133–133·5°, $[\alpha]_D^{16} - 14°$ (*c* 1 in water) (Found: C, 37·8; H, 6·0; N, 6·5; S, 14·2; equiv. wt. 225. $C_7H_{13}NO_6S$ requires C, 37·7; H, 5·9; N, 6·3; S, 14·4%; equiv. wt. 223).

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

(*b*) L-[^{35}S]Cystine. This was prepared from ^{35}S-labelled yeast by the method of Williams & Dawson (1952) as modified by Marsden & Young (1958). It had a specific activity of 108 μc/m-mole and appeared radiochemically pure in the constant-solubility test of Gutmann & Wood (1949).

(*c*) *S*-Ethyl-L-[^{35}S]cysteine. Method 1 described by Thomson *et al.* (1963) for the preparation of the non-radioactive compound was used to prepare *S*-ethyl-L-[^{35}S]cysteine from L-[^{35}S]cystine. It had a specific activity of 51·5 μc/m-mole. The purity was further checked by chromatography in solvents A and B: on scanning for ^{35}S and dipping in ninhydrin only one component was observed on each chromatogram.

(*d*) Ethyl[^{35}S]mercapturic acid. This was prepared by a modification of method 2 of Thomson *et al.* (1963) for the preparation of the non-radioactive compound. *S*-Ethyl-L-[^{35}S]cysteine (0·308 g.) was acetylated in alkaline solution with keten. The Na^+ and OH^- ions were removed from solution by passage down a column of Zeo-Karb 225 resin (50–100 mesh; H^+ form), and the eluate was evaporated under reduced pressure and dried over P_2O_5 and solid NaOH *in vacuo*. The residue was dissolved in 20 ml. of acetone and precipitated by the addition of 0·37 ml. of dicyclohexylamine dissolved in 5 ml. of acetone. Recrystallization of the dicyclohexylammonium salt gave 0·505 g., m.p. 171–171·5°, $[\alpha]_D^{17} - 8°$ (*c* 1·7 in water), specific activity 51·7 μc/m-mole. When chromatograms run in solvents A and B were scanned for ^{35}S and treated with the modified platinum reagent (see below) only one component was detected.

The dicyclohexylammonium salt was too toxic to be administered at the required level for dosing ethylmercapturic acid and it was therefore converted into the sodium salt by passing a solution down a column of Zeo-Karb 225 resin (50–100 mesh; H^+ form), neutralizing the eluate with $NaHCO_3$ and evaporating the solution at 40–45°. The residue was dissolved in water for dosing. Chromatograms of this solution in solvents A and B, when scanned for ^{35}S and treated with the modified platinum reagent, showed only one component, namely ethyl[^{35}S]mercapturic acid.

METHODS

Chromatography. Paper chromatograms were developed overnight on Whatman no. 1 paper by the ascending or descending method with two solvent mixtures: A, butan-1-ol–acetic acid–water (4 : 1 : 5, by vol.); B, butan-1-ol–pyridine–3N-NH_3 solution (4 : 3 : 3, by vol.). Thick paper chromatograms were prepared by the ascending method on Whatman 3MM paper. Two detecting reagents were used. The platinum reagent of Toennies & Kolb (1951) was used to detect bivalent sulphur compounds and sulphoxides. This reagent was also used in a modified form of which 75 ml. contained 0·5 ml. of HCl (sp.gr. 1·18). In this form it allowed a more rapid detection of sulphoxides. Chromatograms were dipped in the reagents, and yellow spots were obtained when chromatograms were warmed, although *S*-alkyl-L-cysteine *S*-oxides sometimes gave white spots. Ninhydrin was used as a 0·2% (w/v) solution in acetone to which 2% of its volume of pyridine had been added immediately before use (Smith, 1960). Chromatograms, which had been treated previously with the modified platinum reagent, were dipped in the ninhydrin reagent to detect *S*-ethyl-L-cysteine. Ethylmercapturic acid sulphone could not be detected with the $K_2Cr_2O_7$–$AgNO_3$ reagent of Knight & Young (1958) or with the platinum reagents. Sometimes it could be detected with 0·02M-$KMnO_4$, but frequently the background colour faded before a spot developed. Detection was best achieved by spraying the paper with 6N-HCl and then heating it, supported between two glass plates, for 3 hr. at 75°. The paper, which was friable after this treatment, was supported on one glass plate and excess of HCl was removed in a stream of air. The ninhydrin reagent was then poured over the paper and *S*-ethyl-L-cysteine *SS*-dioxide, which unlike the corresponding sulphoxide is stable towards acid, was detected as an orange spot.

Radiochromatography. Measured quantities of the urine of animals dosed with radioactive compounds were chromatographed on strips, 1 in. wide, of Whatman no. 1 paper with solvents A and B. Aqueous solutions of ethyl[^{35}S]mercapturic acid or *S*-ethyl-L-[^{35}S]cysteine were chromatographed similarly.

The chromatograms were scanned automatically by using a modification of the apparatus described by Morrison & Young (1959) which permitted the scanning of both sides of the strip simultaneously and allowed an approximate determination of the amount of radioactivity to be made. A pair of Geiger–Müller tubes, set window to window on a common axis, were separated from each other by two metal plates in which slots 1 cm. wide had been cut. The slots were parallel and centred on the axis of the tubes. The chromatogram was drawn between the plates at 30 in./hr. and the output from both tubes was fed into the rate-meter which was connected to the pen-recording milliammeter. The decay time of the rate-meter was set at 12 sec., and this smoothed the recording without significantly broadening

the record of radioactive peaks. The amplitude of the pen-recorder was not a linear function of the output of the rate-meter and therefore a recording chart with a corrected scale was used. The area under a recorded peak of radioactivity was measured in terms of this corrected scale. When scans were made of chromatograms of 0·030, 0·050, 0·105, 0·150 and 0·203 μmole of dicyclohexylammonium ethyl[^{35}S]mercapturate (specific activity 51·7 μc/m-mole) which had been developed in solvent B, the areas of the peaks in arbitrary units less the background values were 1·85, 2·76, 6·29, 8·71 and 11·92 respectively. Similarly, chromatograms of 0·033, 0·050, 0·096, 0·151 and 0·189 μmole of *S*-ethyl-L-[^{35}S]cysteine (specific activity 51·5 μc/m-mole) gave peak areas of 1·63, 3·06, 5·58, 8·83 and 11·92 respectively. Compared with the levels of radioactivity measured the background was sensibly constant.

Detection of ethylmercapturic acid sulphoxide in eluates. Samples (0·1 ml.) were heated with 0·5 ml. of 6N-HCl for 1 hr. in a boiling-water bath. The solution was neutralized with 2N-NaOH and the amino acid present was estimated by the method of Moore & Stein (1954) except that the ninhydrin colour was measured with an EEL colorimeter fitted with an Ilford OGR1 filter.

Enzymic deacetylation. This was brought about by incubating the substrate at 37° for 70 hr. at pH 8 with a pig-kidney deacetylase preparation obtained by the method of Birnbaum, Levintow, Kingsley & Greenstein (1952).

Animals and dosing. Male rats were given a diet of rat cake [J. Murray and Sons (London) Ltd.] and water, and urine was collected separate from faeces during consecutive 24 hr. periods after the dosing. All compounds were injected subcutaneously into the lumbar region of rats that had been lightly anaesthetized with ether.

RESULTS

Metabolism of bromoethane

The ^{35}S-labelled yeast was fed as a 5% (w/w) mixture with the diet described by Maw (1953) to four rats each weighing about 170 g. Each rat received 20 g./day for 3 days. On the fourth day they were given the diet from which ^{35}S-labelled yeast had been omitted, and two were injected with 0·25 g. of bromoethane as a 40% (w/v) solution in arachis oil; the other pair were used as controls and given arachis oil alone.

Chromatograms of the urine were developed in two solvent systems, scanned for radioactivity and then treated with the platinum reagent (Table 1). The radiochromatograms showed the presence of two major components in approximately equal amounts. The spot with the higher R_F in both solvents was shown by Thomson, Maw & Young (1958) to be due to ethylmercapturic acid and this compound was isolated by Thomson *et al.* (1963). The material with R_F values 0·47 and 0·29 in solvents A and B respectively was eluted with water from the appropriate zone of thick paper chromatograms on which 0·25 ml. of urine had been applied to strips of paper 7·5 cm. wide. The eluates were evaporated under reduced pressure until the concentration of the solute was equivalent to about 40% of its concentration in the original urine. Chromatography of each eluate in the solvent other than that in which its solute was first separated showed that they contained radioactive material with identical chromatographic properties. Samples of each eluate were subjected to hydrolysis, reduction with thioglycollic acid, and enzymic deacetylation, and the reaction products were compared chromatographically with those obtained by the same treatment of samples of authentic ethylmercapturic acid sulphoxide. When a sample was refluxed with an equal volume of 12N-hydrochloric acid for 2·5 hr., the reaction mixture contained material identical with *S*-ethyl-L-cysteine. After 1 ml. of eluate had been adjusted to pH 3 and

Table 1. *Radiochromatographic examination of urine excreted by rats in the first 24 hr. period after they had received a diet containing ^{35}S-labelled yeast and had then been injected subcutaneously with bromoethane*

Details are given in the text. The R_F values reported for synthetic compounds were obtained by chemical tests; all other R_F values are for peaks on radiochromatograms.

	R_F							
Solvent mixture ...	A	B	A	B	A	B	A	B
S-Ethyl-L-cysteine	—	—	0·45*	0·45*	—	—	—	—
Ethylmercapturic acid	—	—	—	—	0·78†	0·52†	—	—
S-Ethyl-L-cysteine *S*-oxide	—	—	—	—	—	—	0·18*	0·21*
			After acid hydrolysis		After reduction		After deacetylation	
Urine	0·77†	0·51†	—	—	—	—	—	—
	0·47‡	0·29‡	—	—	—	—	—	—
Eluate from thick paper chromatograms	0·46‡	0·31‡	0·45*	0·45*	0·77	0·51†	0·18*	0·20*
(+)-Ethylmercapturic acid sulphoxide	0·47‡	0·30‡	0·44*	0·46*	—	0·50†	—	—
(−)-Ethylmercapturic acid sulphoxide	0·44‡	0·33‡	0·45*	0·45*	—	0·50†	0·18*	0·21*

* Reaction with the platinum reagent and with the ninhydrin reagent.
† Rapid reaction with the platinum reagent. ‡ Slow reaction with the platinum reagent.

heated with 10 μl. of thioglycollic acid under nitrogen for 5 hr. at 80°, the presence of ethylmercapturic acid could be demonstrated. After enzymic deacetylation *S*-ethyl-L-cysteine *S*-oxide was present. The results in Table 1 show that the material eluted from chromatograms behaved identically with the synthetic samples of ethylmercapturic acid sulphoxide. No compounds corresponding to ethylmercapturic acid or to its sulphoxide were detected in the urine of animals that had received arachis oil alone.

The fact that the ethylmercapturic acid sulphoxide was not formed from ethylmercapturic acid after excretion of the latter was shown by the following experiment. Ethylmercapturic acid was eluted from thick paper chromatograms and the eluate was evaporated at 45° under reduced pressure. The mercapturic acid was dissolved in normal urine so that its concentration was 2–3 times that in the urine excreted after dosing with bromoethane. After 24 hr. at room temperature chromatograms of the solution were prepared and scanned for radioactivity. Only ethylmercapturic acid was detected after chromatography with solvent A; a trace of material with the same R_F as ethylmercapturic acid sulphoxide was detected after chromatography with solvent B.

Metabolism of S-*ethyl*-L-*cysteine and ethylmercapturic acid*

Chromatographic and radiochromatographic studies. Four male rats (body wt. 180–200 g.) were housed in separate metabolism cages. Two received 1·0 and 1·5 ml. respectively of 0·36 M-*S*-ethyl-L-[^{35}S]cysteine in M-sodium hydrogen carbonate and the others received 1·0 and 1·7 ml. respectively of 0·33 M-sodium ethyl[^{35}S]mercapturate in water. The urines excreted in the first 24 hr. were pH 8·0 from the pair that had received *S*-ethyl-L-[^{35}S]cysteine and pH 7·2 from the pair dosed with ethyl[^{35}S]mercapturic acid. The cages and urine–faeces separators were washed down with water, diluting each urine to 20 ml. Immediately after collection 20 μl. samples of the diluted urine were chromatographed in solvents A and B. The chromatograms were scanned for radioactivity and then treated with the modified platinum reagent followed by ninhydrin.

All four urine samples gave similar chromatographic patterns, as shown in Table 2. When treated with chemical-detecting agents, chromatograms of urine excreted in the second and third 24 hr. periods by the dosed animals, and by control animals injected with water or M-sodium hydrogen carbonate, showed no spots comparable with those observed on chromatograms of the urine excreted in the first 24 hr. period by the dosed animals. On the basis of R_F values and detection with the modified platinum reagent the fastest-running major component was identified as ethylmercapturic acid. The isolation of this compound from the urine of rats that had been dosed with *S*-ethyl-L-cysteine was described by Thomson *et al.* (1963). The other major metabolite, with R_F values about 0·5 and 0·3 in solvents A and B respectively, had the same R_F values as ethylmercapturic acid sulphoxide and was detected with the modified platinum reagent as a yellow spot.

Further evidence that this metabolite was ethylmercapturic acid sulphoxide was obtained by chromatographing, with solvent B, samples of urine that had been hydrolysed with hydrochloric acid or treated with thioglycollic acid. After acid hydrolysis (0·5 ml. of urine and 0·5 ml. of 12 N-hydrochloric acid heated in a sealed tube for 4 hr. at 90°) one major radioactive spot with R_F 0·37 and minor spots with R_F 0·05 and R_F 0·53 were detected. The major component was also detected with the modified platinum reagent and with ninhydrin and ran identically with *S*-ethyl-L-cysteine. Its total radioactivity was much greater than could have been derived from the ethylmercapturic acid present. When urine had been reduced [0·5 ml. of

Table 2. *Radiochromatographic examination of the urine excreted by rats in the first 24 hr. period after they had been injected subcutaneously with* S-*ethyl*-L-[^{35}S]*cysteine and sodium ethyl*[^{35}S]*mercapturate*

Details are given in the text. The R_F values of all the radioactive metabolites detected are given; the R_F values of the reference compounds were obtained by chemical tests. The percentage conversion of administered compounds to metabolites is given in parentheses.

Solvent mixture ...	R_F A				R_F B		
Urine after dosing with *S*-ethyl-L-[^{35}S]cysteine	0·05	0·21	0·48*	0·79†	0·05 (5)	0·32* (36)	0·54† (26)
Urine after dosing with sodium ethyl[^{35}S]mercapturate	0·05	0·19	0·46*	0·78†	0·04 (5)	0·29* (30)	0·53† (34)
Ethylmercapturic acid	—	—	—	0·77†	—	—	0·53†
(+)-Ethylmercapturic acid sulphoxide	—	—	0·45*	—	—	0·28*	—

* Detected as a yellow spot with the modified platinum reagent.
† Detected as a white spot with the modified platinum reagent.

urine and 0·1 ml. of 7% (v/v) thioglycollic acid heated in a sealed tube at 90° for 4 hr.] a radioactive spot with R_F 0·49, which was also detected with the modified platinum reagent, was found together with a minor radioactive spot with R_F 0·05. The spot with R_F 0·49 ran identically with ethylmercapturic acid and its total radioactivity was greater than that of the ethylmercapturic acid originally present. The production of *S*-ethyl-L-cysteine on acid hydrolysis and ethylmercapturic acid on reduction are properties of both isomers of ethylmercapturic acid sulphoxide that have been described in the present paper. Confirmation of the excretion of ethylmercapturic acid sulphoxide after dosing with *S*-ethyl-L-cysteine was obtained by its isolation (see below).

The percentage conversion of *S*-ethyl-L-[^{35}S]cysteine and ethyl[^{35}S]mercapturic acid into the various metabolites was calculated from the areas of the peaks of radioactivity obtained on scanning chromatograms that had been prepared with solvent B. Conversion into ethyl[^{35}S]mercapturic acid sulphoxide of 36% of the *S*-ethyl-L-[^{35}S]cysteine and of 30% of the ethylmercapturic acid was observed (Table 2). When the experiments were repeated similar radiochromatograms were obtained and the corresponding conversions were 33% and 30%. *S*-Ethyl-L-[^{35}S]cysteine and ethyl[^{35}S]mercapturic acid gave minor unidentified radioactive metabolites near the origin on chromatograms run with solvent B and near the origin and at R_F about 0·20 with solvent A. They were not ethylmercapturic acid sulphone, *S*-ethyl-L-cysteine *S*-oxide or *S*-ethyl-L-cysteine *SS*-dioxide. Chromatographic comparison with authentic samples of these compounds showed that ethylmercapturic acid sulphone appeared at R_F 0·52 and R_F 0·44 after chromatography in solvents A and B respectively, and that on acid hydrolysis it broke down to give *S*-ethyl-L-cysteine *SS*-dioxide which ran with R_F values 0·20 and 0·38 in solvents A and B respectively. The metabolite with R_F about 0·20 in solvent A was not *S*-ethyl-L-cysteine *SS*-dioxide for it was destroyed by acid. It was not *S*-ethyl-L-cysteine *S*-oxide, for this compound had a lower R_F in solvent A and had R_F 0·14 in solvent B, at which R_F no radioactive metabolite was found.

When normal rat urine, containing added ethylmercapturic acid at a concentration of 49 mM and adjusted to pH 5·0, 7·2 or 8·2, was stored at room temperature for 22 hr. and chromatographed with solvents A and B, only unchanged ethylmercapturic acid was detected. Similar experiments were carried out with urine containing dicyclohexylammonium ethyl[^{35}S]mercapturate at a concentration of 47 mM and *S*-ethyl-L-[^{35}S]cysteine at 60 mM, and no sulphoxide was detected even though scanning for radioactive compounds was more sensitive than chemical detection. It is apparent, therefore, that ethylmercapturic acid sulphoxide was not produced as an artifact by the oxidation of ethylmercapturic acid during collection and chromatography of the urine.

Isolation of (+)-ethylmercapturic acid sulphoxide after the administration of S*-ethyl-*L*-cysteine.* Four male rats (body weight 170–210 g.) were each given 1·5 ml. of 0·34 M-*S*-ethyl-L-cysteine in M-sodium hydrogen carbonate by subcutaneous injection. Urine (volume 45 ml.; pH 8·0) was collected during the first 24 hr. period after the dosing. This was subjected to the procedure developed by Thomson *et al.* (1963) for the isolation of ethylmercapturic acid from the urine of rats that had been dosed with *S*-ethyl-L-cysteine, and which involved the adsorption of acids on the anion-exchange resin Amberlite CG-400 (formate form) followed by gradient elution with formate buffer, pH 4·1. The sulphoxide was detected in the eluate by comparing the ninhydrin reaction of samples of each fraction before and after hydrolysis. A band of material was detected preceding ethylmercapturic acid, and the fractions containing this band were combined and worked up as described for the isolation of ethylmercapturic acid. From 27 ml. of urine 0·128 g. of (+)-dicyclohexylammonium ethylmercapturate sulphoxide was obtained, m.p. 164–166° (decomp.), unchanged on admixture with an authentic sample and depressed to 158–159° when mixed with the (−)-isomer, $[\alpha]_D^{16}+17°$ (*c* 1·5 in water) (Found: C, 58·5; H, 9·0; N, 6·9; S, 7·7%). The equivalent weight determined by the method of Thomson *et al.* (1963) was 394 (calc. 388). The amount of (+)-dicyclohexylammonium ethylmercapturate sulphoxide isolated from the urine of the dosed rats represented a conversion of 27% of the *S*-ethyl-L-cysteine administered. That obtained from the urine in a similar experiment with another group of rats represented a conversion of 21%.

When samples were chromatographed they behaved identically with authentic samples of (+)-dicyclohexylammonium ethylmercapturate sulphoxide. Both the isolated and authentic material gave *S*-ethyl-L-cysteine after acid decomposition, and when reduced with thioglycollic acid they gave ethylmercapturic acid. The isolated compound had an infrared-absorption spectrum identical with that of synthetic (+)-dicyclohexylammonium ethylmercapturate sulphoxide.

The efficiency of the isolation procedure was checked by measuring the recovery of synthetic ethylmercapturic acid and its sulphoxides added to normal rat urine. When 0·099 g. of ethylmercapturic acid and 0·110 g. of (−)-ethylmercapturic acid sulphoxide were added to 16 ml. of urine and kept for 22 hr., 0·143 g. of dicyclohexylammonium ethylmercapturate and 0·166 g. of (−)-dicyclohexyl-

ammonium ethylmercapturate sulphoxide were obtained. The latter had m.p. 166–167° (decomp.), $[\alpha]_D^{16} - 39°$ (*c* 1·5 in water). This represents a recovery of 74% of the mercapturic acid and 81% of the sulphoxide. In an experiment in which 0·093 g. of ethylmercapturic acid, 0·022 g. of dicyclohexylammonium ethyl[^{35}S]mercapturate (specific activity 51·7 μc/m-mole) and 0·191 g. of (+)-dicyclohexylammonium ethylmercapturate sulphoxide were added to normal rat urine, 0·160 g. of dicyclohexylammonium ethylmercapturate and 0·169 g. of (+)-dicyclohexylammonium ethylmercapturate sulphoxide, m.p. 163–165° (decomp.), $[\alpha]_D^{16} + 17°$ (*c* 1·5 in water), specific activity 0·105 μc/m-mole, were obtained. This represents a recovery of 78% of the mercapturic acid and 89% of the sulphoxide, and also indicates that only 1·9% of the sulphoxide isolated was derived from ethylmercapturic acid during the isolation.

Metabolism of S-*ethyl*-L-*cysteine* S-*oxide and* (+)- *and* (−)-*ethylmercapturic acid sulphoxide*

Two rats (body weight 180 g.) were each dosed with 1·5 ml. of 0·20 M-*S*-ethyl-L-cysteine *S*-oxide in water. The urine excreted in the first 24 hr. period after dosing (21 ml.; pH 7·2) was chromatographed, and after application of the modified platinum reagent only ethylmercapturic acid sulphoxide was detected. Chromatography of hydrolysed urine and of urine subjected to reduction with thioglycollic acid revealed the presence of ethylcysteine and ethylmercapturic acid respectively. To investigate the possibility that small quantities of ethylmercapturic acid were also excreted, 0·3 ml. of urine was chromatographed in solvent A on a strip of paper 30 cm. wide. Ethylmercapturic acid sulphoxide was detected at R_F values not exceeding 0·48 on test strips cut from the chromatogram. That part of the chromatogram between R_F 0·65 and R_F 0·90 was cut out and eluted with water, and the eluate was evaporated over phosphorus pentoxide under reduced pressure to give a residue which was dissolved in 0·4 ml. of water. Chromatography of 50 μl. of this solution and detection with the modified platinum reagent gave a chromatogram, similar to that obtained with normal urine taken through a similar procedure, with a very faint spot at R_F 0·80 in solvent A. A sample of the eluate was hydrolysed, chromatographed in solvent A and the chromatogram treated with ninhydrin. A strong amino acid pattern was obtained similar to that from normal urine, but with no reaction at the R_F of *S*-ethyl-L-cysteine. Similar results were obtained when rats were dosed with (+)- or (−)-ethylmercapturic acid sulphoxide.

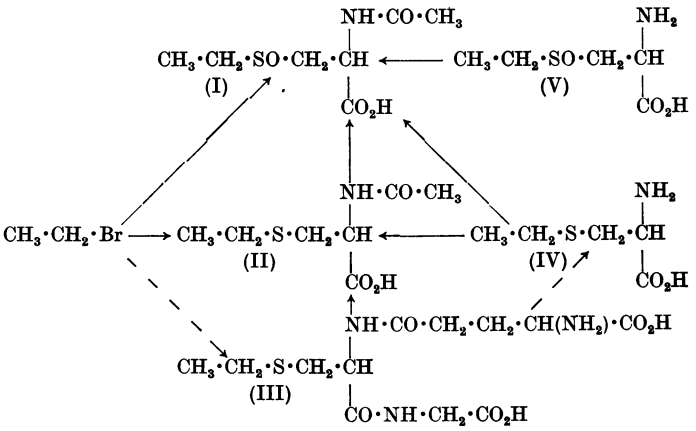
When 1·4 mg. of ethylmercapturic acid was added to 20 ml. of normal urine and the urine was taken through the above procedure, ethylmercapturic acid was detected on chromatograms of the eluate and ethylcysteine was detected on chromatograms of the hydrolysed eluate. It therefore appears that, if ethylmercapturic acid was excreted after the administration of *S*-ethyl-L-cysteine *S*-oxide, the amount was equivalent to less than 1% of the material given.

DISCUSSION

The ready oxidation of methionine (Dent, 1948) and *S*-methyl-L-cysteine (Arnold & Thompson, 1962) to the corresponding sulphoxides has been reported, and the possibility arose, therefore, that the ethylmercapturic acid sulphoxide found in the present work was an artifact. A sensitive radiochromatographic method, however, gave no evidence for the oxidation of ethyl[^{35}S]mercapturic acid or *S*-ethyl-L-[^{35}S]cysteine during chromatography of solutions in urine. Further, after the administration of *S*-ethyl-L-cysteine to rats the isolation of ethylmercapturic acid sulphoxide from their urine, by a method that in control experiments gave recoveries of 74 and 78% of ethylmercapturic acid and 81 and 89% of ethylmercapturic acid sulphoxide, indicated that the (+)-isomer was the principal if not the only isomer present. Although partial asymmetric oxidation of methionine (Lavine, 1947) and methionine methyl ester (Iselin, 1961) by chemical methods has been observed, it has been shown that only a very small proportion of (+)-ethylmercapturic acid sulphoxide was derived from ethylmercapturic acid during the isolation procedure. In control experiments, not only were high recoveries of ethylmercapturic acid and its sulphoxide obtained, but the radioactivity of ethylmercapturic acid sulphoxide recovered from urine to which ethyl[^{35}S]mercapturic acid had been added indicated that not more than 1·9% of the isolated sulphoxide was derived from the mercapturic acid.

The absolute configuration of (+)-*S*-methyl-L-cysteine *S*-oxide is known and it has been suggested, on the basis of optical rotations, that *S*-allyl- and other naturally occurring (+)-*S*-alkyl-L-cysteine *S*-oxides are sterically related to it (Klyne, 1960). (+)-Ethylmercapturic acid sulphoxide may also have the same configuration.

The present work appears to provide the only evidence so far for the formation of a mercapturic acid sulphoxide *in vivo*. Although mercapturic acids are formed *in vivo* from certain higher alkyl halides, the metabolites accompanying them do not include sulphoxides (James, 1961). No evidence has been obtained for the oxidation *in vivo* to the sulphone of *S*-ethyl-L-cysteine, ethylmercapturic acid or its sulphoxides, but Roberts & Warwick (1958) have adduced evidence for the excretion of



Scheme 1. Biosynthesis of ethylmercapturic acid sulphoxide. Conversions demonstrated in the animal body are shown by solid arrows and possible conversions are indicated by broken arrows.

ethylcysteine sulphone in the urine of rats after the administration of ethylcysteine. In the present work two minor metabolites of *S*-ethyl-L-cysteine and of ethylmercapturic acid have been observed radiochromatographically, one near the origin and the other with R_F about 0·2 in solvent A. These have not been identified, but as ethanethiol is formed by the action of liver enzymes on *S*-ethyl-L-cysteine (Binkley, 1950) it is possible that this compound or its metabolites (cf. Snow, 1957) were present in the urine.

The reactions that have been observed *in vivo* are included in Scheme 1, which also shows the possible routes for the formation of ethylmercapturic acid sulphoxide (I) from bromoethane.

Roberts & Warwick (1958) demonstrated the formation of ethylmercapturic acid (II) from *S*-ethylglutathione (III), and they also reported the presence of a second metabolite which they did not identify. *S*-Ethyl-L-cysteine (IV) gives rise *in vivo* to both ethylmercapturic acid (Thomson *et al.* 1963) and ethylmercapturic acid sulphoxide. Ethylmercapturic acid is also converted into ethylmercapturic acid sulphoxide, and acetylation followed by oxidation is therefore a metabolic pathway for the formation of ethylmercapturic acid sulphoxide from *S*-ethyl-L-cysteine. The possibility cannot be ruled out, however, that there is an alternative route, namely oxidation followed by acetylation, for the formation of ethylmercapturic acid from *S*-ethyl-L-cysteine *S*-oxide (V) *in vivo* has also been demonstrated.

SUMMARY

1. (+)-Ethylmercapturic acid sulphoxide, i.e. (+)-*N*-acetyl-*S*-ethyl-L-cysteine *S*-oxide, has been prepared as the dicyclohexylammonium salt, and (−)-ethylmercapturic acid sulphoxide has been prepared as both the free acid and the dicyclohexylammonium salt. Some properties of these compounds have been reported.

2. *S*-Ethyl-L-cysteine *S*-oxide, *S*-ethyl-L-cysteine *SS*-dioxide and *N*-acetyl-*S*-ethyl-L-cysteine *SS*-dioxide have also been prepared.

3. (+)-Ethylmercapturic acid sulphoxide has been isolated as the dicyclohexylammonium salt from the urine of rats injected subcutaneously with *S*-ethyl-L-cysteine.

4. The excretion of ethylmercapturic acid sulphoxide in the urine of rats that have been injected subcutaneously with bromoethane or ethylmercapturic acid has been demonstrated by radiochromatographic methods, and its excretion after the injection of *S*-ethyl-L-cysteine *S*-oxide has been shown by paper chromatography.

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Biochem. J. (1964) **90**, 596

Studies on Carbohydrate-Metabolizing Enzymes

11. THE HYDROLYSIS OF LICHENIN BY ENZYME PREPARATIONS FROM MALTED BARLEY AND *RHIZOPUS ARRHIZUS**

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Lichenin, the reserve carbohydrate of Iceland moss (*Cetraria islandica*), is an essentially linear polymer of β-D-glucopyranose containing 30% of (1→3)- and 70% of (1→4)-linkages (Chanda, Hirst & Manners, 1957). The sequence of the linkages has not been rigidly established. A partial acid hydrolysate contained the disaccharides cellobiose and laminaribiose, and the trisaccharides cellotriose, 4^2-β-glucosyl-laminaribiose and 3^2-β-glucosylcellobiose (Peat, Whelan & Roberts, 1957; for nomenclature of oligosaccharides see Whelan, 1960). Since laminaritriose was absent, it was suggested that the molecule consisted of cellotriose units joined together by (1→3)-linkages. A structure of lichenin proposed by Peat *et al.* (1957) is shown in Fig. 1.

During the period 1920–40, many workers reported 'lichenase' activity in enzyme preparations from a wide variety of biological sources (for a review, see Pigman, 1951). In view of the similarity in structure of lichenin and cellulose (although the presence of (1→3)-linkages in lichenin was not then known), and in their behaviour with various enzyme preparations, it was suggested that 'lichenase' and 'cellulase' were identical or closely related enzymes. For example, Otto (1929) could not separate the two activities in a malted-barley preparation. However, later workers (e.g. Freudenberg & Ploetz, 1939) obtained some separation of

* Part 10: Anderson, Cunningham & Manners (1964).

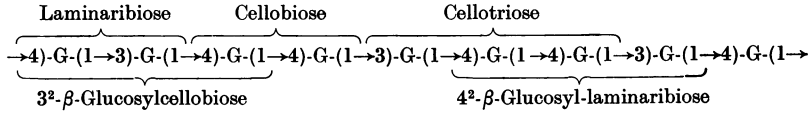

Fig. 1. Structure of lichenin proposed by Peat, Whelan & Roberts (1957), on the basis of partial acid hydrolysis studies. G, β-Glucopyranose residue. The oligosaccharide fragments are also indicated.