The Metabolism of Phenethyl Bromide, Styrene and Styrene Oxide in the Rabbit and Rat

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1. The chief sulphur-containing metabolite of styrene and styrene oxide in the rabbit and rat is chromatographically identical with *N*-acetyl-*S*-(β -hydroxyphenethyl)-L-cysteine and this compound is also formed, together with *N*-acetyl-*S*-phenethyl-L-cysteine, as a metabolite of phenethyl bromide. 2. The amounts of the phenethylmercapturic acid and its hydroxy derivative excreted in the urine of animals dosed with phenethyl bromide, styrene, styrene oxide, phenyl glycol, *S*-phenylethylcysteine and phenethylmercapturic acid have been determined. 3. Liver slices convert phenethylcysteine and phenethylmercapturic acid into *N*-acetyl-*S*-(β -hydroxyphenethyl)-L-cysteine. 4. Methods for the determination by gas-liquid chromatography of mandelic acid and hippuric acid, which are metabolites of some of the compounds studied, are described.

Many examples of the excretion of *N*-acetyl-*S*-alkylcysteines (alkylmercapturic acids) by animals dosed with halogenoalkanes have been described (e.g. Bray & James, 1958; Thomson, Maw & Young, 1958; Grenby & Young, 1960; Bray, Caygill, James & Wood, 1964) and in some cases the formation of hydroxyalkylmercapturic acids has also been reported (e.g. Barnsley, 1964; Barnsley, Grenby & Young, 1966; James & Jeffery, 1964).

It was tentatively suggested by James & Jeffery (1964) that the hydroxyalkyl derivatives might be formed in the way shown in Scheme 1. In this scheme the hydroxyl group is formed when an intermediate epoxide (III) combines with GSH to give compound (IV). The possibility that the initial reaction consists of the conjugation of halogenoalkane (I) with GSH and that hydroxylation of the alkyl chain occurs at a later stage cannot be excluded. Several enzymes have been described that catalyse the conjugation of mercapturic acid precursors with GSH. Of these the glutathione

S-epoxidetransferase described by Boyland & Williams (1965) would catalyse the reaction between the epoxide (III) and GSH and lead to the formation of a hydroxymercapturic acid (VI) as indicated; the glutathione *S*-alkyltransferase described by Johnson (1966) would catalyse the reaction between the halogenoalkane (I) and GSH and lead to the formation of an alkylmercapturic acid only, unless hydroxylation occurred after the initial reaction. Boyland & Chasseaud (1966) have described a further enzyme that catalyses the reaction between GSH and certain compounds containing an activated double bond and this again would lead to the formation of a mercapturic acid unless a hydroxyl group was introduced at a later stage.

It seemed possible that a re-examination of the metabolism of phenethyl bromide (I, R=C₆H₅), styrene (II, R=C₆H₅) and styrene oxide (III, R=C₆H₅) might afford further information about the formation of hydroxymercapturic acids. Phenethyl bromide was shown to be a precursor of

Scheme 1. Possible pathway for the formation of hydroxyalkylmercapturic acids.

phenethylmercapturic acid in the rabbit (Bray, James & Thorpe, 1958), but the formation of a hydroxyphenethylmercapturic acid was not detected. The metabolism of styrene and styrene oxide was described by Smith, Smithies & Williams (1954) and by El Masri, Smith & Williams (1958). The alkali-hydrolysed urine of rabbits dosed with styrene showed an increased uptake of iodine, indicating the possible formation of mercapturic acid, but this possibility was not investigated in animals dosed with styrene oxide. El Masri *et al.* (1958) showed that mandelic acid (XIII, Scheme 2) is a metabolite of styrene and styrene oxide and recorded the amount isolated. In the present work a method for the determination of mandelic acid has been developed. Styrene oxide may be an intermediate in the conversion of styrene into mandelic acid, and if the epoxide pathway leads to the formation of hydroxymercapturic acids then in this group of compounds some correlation between the formation of hydroxyphenethylmercapturic acid and mandelic acid should exist.

MATERIALS

All melting points are uncorrected. Carbon, hydrogen and nitrogen microanalyses were carried out by F. and E. Pascher, Bonn, W. Germany. Sulphur was determined by the flask method (see Macdonald, 1961) and titration with $\text{Ba}(\text{ClO}_4)_2$.

Phenethyl bromide, styrene, styrene oxide and phenyl glycol (styrene glycol) were purchased (Koch-Light Laboratories Ltd., Colnbrook, Bucks.). *S*-Phenethyl-L-cysteine was prepared and acetylated to give *N*-acetyl-*S*-phenethyl-L-cysteine as described by Bray *et al.* (1958). This mercapturic acid was also obtained by the interaction of *N*-acetyl-L-cysteine and phenethyl bromide according to the method of Boyland & Sims (1965). The product had m.p. 130° and was chromatographically identical with the phenethylmercapturic acid prepared by Bray *et al.* (1958).

N-Acetyl-*S*- β -hydroxyphenethyl-L-cysteine. *N*-Acetyl-L-cysteine (2.5 g.), styrene oxide (1.8 ml.) and NaHCO_3 (4 g.) in aq. 50% (v/v) acetone (250 ml.) were heated under reflux for 6 hr. The acetone was removed by distillation under reduced pressure, and the remaining solution (about 100 ml.) was then acidified with HCl and extracted with ether. The yellow gum left after evaporation of the ether extract was dissolved in acetone, and a solution of dicyclohexylamine in acetone was added until the solution was just alkaline. The crystals (1.6 g.; yield 25%) of the dicyclohexylammonium salt of *N*-acetyl-*S*- β -hydroxyphenethylmercapturic acid that separated had m.p. 175° after recrystallization from aqueous ethanol (Found: C, 65.1; H, 8.2; N, 5.5; S, 6.85%; equiv.wt. 464 ± 4 . $\text{C}_{25}\text{H}_{40}\text{O}_4\text{N}_2\text{S}$ requires C, 64.7; H, 8.6; N, 6.0; S, 6.9%; equiv.wt. 464).

[1- ^{14}C]Styrene oxide. Phenylacetic acid (0.125 mole) containing 0.1 mc as phenyl[1- ^{14}C]acetic acid (The Radiochemical Centre, Amersham, Bucks.) was reduced to 2-phenylethanol with LiAlH_4 (Nyström & Brown, 1947). The crude product was converted into styrene by the method of Hodnett & Jensen (1960) with the addition of sulphur to prevent polymerization. The product was

purified by distillation (yield 88%), b.p. 146° [Hodnett & Jensen (1960) give b.p. 146°]; it was characterized by conversion into 1,2-dibromo-1-phenylethane, m.p. 73–74° [Heilbron & Bunbury (1943) give m.p. 73°]. Styrene (5 ml.) was converted into styrene oxide by the action of perbenzoic acid (Hickinbottom, 1948).

^{35}S -labelled yeast. Yeast was grown on a medium containing sodium [^{35}S]sulphate as described by Williams & Dawson (1952).

METHODS

Animals, diet and dosage

Doe rabbits (2.5 kg.) and rats (200–250 g.) were maintained as described by Bray, Franklin & James (1959). The rats used for the experiments with ^{35}S were maintained on a mixture of powdered rat cake and dried yeast (7:3, w/w). ^{35}S -labelled yeast (2 g.) was incorporated into the diet on the day before dosing and the subsequent 24 hr. sample of normal urine collected; a further 1 g. was given in the food on the following day about 3 hr. before dosing, and the urine collected after 24 hr. All doses were administered by stomach tube and the amounts are recorded in Table 4.

Chromatography

Paper chromatography. Whatman 3MM paper was used and the chromatograms were developed for about 1 hr. by the descending method. The solvent mixtures A, P, C and D described in Table 1 were used. A better separation was achieved when the solvents were allowed to run off the edge of the paper. The distances substances moved were compared with that moved by phenethylmercapturic acid, which was taken as 1.0 in all the solvent systems. The detecting reagents used were the $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 reagent of Knight & Young (1958) and the platinum reagent of Toennies & Kolb (1951) as modified by Barnsley, Thomson & Young (1964) for cysteine derivatives, the 8-hydroxyquinoline sulphate- ZnSO_4 reagent described by Pesez & Ferrero (1957) for acids (mercapturic acids, mandelic acid and hippuric acid run in solvent A, C or D), the ninhydrin reagent of Moffat & Lytle (1959) for free amino acids, and diazotized *p*-nitroaniline followed by 20% Na_2CO_3 solution for phenols (Bray & Thorpe, 1954).

Thin-layer chromatography. Glass plates coated with a layer of silica gel G (E. Merck A.-G., Darmstadt, W. Germany) 250 μ thick were used. The chromatograms were developed by the ascending method with solvent E (Table 1) for *N*-acetylcysteine derivatives and solvent F for amino acids.

Gas-liquid chromatography. The column used was that described for the determination of hippuric acid by James, Waring & White (1967). The reference compounds were esterified with diazomethane, prepared from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide (British Drug Houses Ltd., Poole, Dorset). *N*-Acetyl-*S*-(β -hydroxyphenethyl)-L-cysteine was liberated from its dicyclohexylammonium salt by passing down a column of Zeo-Karb 225 and then methylated. The retention times at 218° are given in Table 1. It was necessary to separate the metabolites before application to the column. The 24 hr. urine of two dosed rats was acidified to pH 2 and extracted overnight with ether. The extract was evaporated, and the residue was dissolved in solvent E and applied to a silica gel G column (8 cm. $\times$ 1 cm.) and eluted with solvent E. Fractions

Table 1. *Relative rates of movement of reference compounds*

For paper and thin-layer chromatography the rate of movement of the compounds is related to that of phenethylmercapturic acid, which is taken as 1.0 in all solvents. Solvent mixtures: A, butan-1-ol-ethanol-aq. NH_3 (sp.gr. 0.88)-water (10:10:1:4, by vol.); B, butan-1-ol-water-acetic acid-butyl acetate (24:10:5:2, by vol.); C, butan-2-one saturated with aq. 2N- NH_3 ; D, butan-1-ol saturated with aq. 2N- NH_3 ; E, chloroform-benzyl alcohol-acetic acid (70:30:3, by vol.); F, butan-1-ol-acetic acid-water (2:1:1, by vol.). The methyl esters were used for gas-liquid chromatography. Detecting reagents (for details see the text): a, $\text{K}_2\text{Cr}_2\text{O}_7\text{-AgNO}_3$; b, platinum reagent; c, 8-hydroxyquinoline; d, ninhydrin; e, diazotized *p*-nitroaniline.

Compound	Detecting reagent	Relative rate of movement						Retention time (min. at 218°) Gas-liquid chromatography
		Paper chromatography in solvent				Thin-layer chromatography in solvent		
		A	B	C	D	E	F	
<i>N</i> -Acetyl- <i>S</i> -phenethyl-L-cysteine	<i>a, b, c</i>	1.0	1.0	1.0	1.0	1.0	1.0	5.0
<i>N</i> -Acetyl- <i>S</i> -(β -hydroxyphenethyl)-L-cysteine	<i>a, b, c</i>	0.77	0.90	0.47	0.50	0.66	1.02	10.3
<i>S</i> -Phenethyl-L-cysteine	<i>a, b, d</i>	0.85	0.66	0.97	1.0	0.74	0.96	—
Mandelic acid	<i>c</i>	0.73	0.92	0.44	0.54	0.17	0.93	< 1.21
Hippuric acid	<i>c</i>	—	—	—	—	—	—	1.21
Unidentified metabolite	<i>a, b</i>	0.72	—	0.23	0.43	—	—	—

(0.5 ml.) were collected and examined by thin-layer chromatography with solvent E and running phenethyl- and β -hydroxyphenethyl-mercapturic acid as markers. Appropriate fractions were combined, evaporated and methylated, and samples were applied to the column of the gas chromatograph.

Determination of metabolites in urine

Glucosiduronic acid. The modified naphtharesorcinol method described by Bray, Humphris, Thorpe, White & Wood (1952) was used except that the colours were read on an EEL colorimeter with filter 607.

Ethereal sulphate. This was determined by the method of Folin (1905-06).

Mercapturic acid. (a) Nitroprusside method. This was used as described by Bray *et al.* (1958). A calibration curve was constructed with the dicyclohexylammonium salt of β -hydroxyphenethylmercapturic acid. This method is not specific and results are recorded in Table 4 as 'apparent mercapturic acid'.

(b) Chromatographic method. This was as described by Bray *et al.* (1964) except that detection was by dipping in the chloroplatinate reagent. Calibration curves were constructed with phenethylmercapturic acid and the dicyclohexylammonium salt of β -hydroxyphenethylmercapturic acid. When the two mercapturic acids were present in markedly different concentrations it was necessary to carry out a separate determination for each to get suitable areas for comparison with the calibration curves.

Phenethylcysteine. Chromatograms were prepared as for the determination of the mercapturic acids and sprayed with the ninhydrin reagent. The intensity of the colour and area of the spots were compared with those given by known amounts of phenethylcysteine.

Mandelic acid. This was determined by gas-liquid chromatography of the methyl ester by using a Pye argon chromatograph with a β -ionization detector. The column

(140 cm. $\times$ 5 mm.) consisted of Celite (80-100 mesh) coated with 25% Edwards Hivac silicone grease and was operated at 130°. The carrier gas was argon (135 ml./min.). The retention time for methyl mandelate was 13.3 min. and that for methyl *p*-chlorobenzoate, which was used as an internal standard, was 10 min.

A calibration curve was obtained as follows. Weighed amounts of mandelic acid and *p*-chlorobenzoic acid were dissolved in ether and esterified with diazomethane. The esters were made up to known volumes, samples of the solutions giving amounts in the range 10-100 μg . were applied to the column and the areas of the peaks were measured. Good agreement between the areas of the peaks was obtained over the range 0-50 μg ., and at 100 μg . the error was still less than 10%.

The preparation of samples from urine was carried out as follows. The urine was collected for 24 hr. and the volume made up to 500 ml. Duplicate samples (20 ml.) were adjusted to pH 2 and continuously extracted with ether for 6 hr. The extracts were dried over anhydrous CaSO_4 and then esterified by the addition of excess of ethereal diazomethane. Excess of ether and diazomethane were evaporated and the residual ether made up to 15 or 20 ml. in ether. Samples of these solutions were applied to the column together with a known amount of methyl *p*-chlorobenzoate. The areas of the peaks were measured, and since the amounts of methyl *p*-chlorobenzoate applied to the column were known the amounts of methyl mandelate were calculated by simple proportion. The urine collected the day before dosing and the urines collected for 3 days after the dose were analysed. No peak corresponding to methyl mandelate was detected in samples from normal urine or from the urine collected on the second or third day after dosing. The recovery of mandelic acid added to urine was $97 \pm 2\%$.

Hippuric acid. This was determined by the method of James *et al.* (1967). The recovery of hippuric acid added to normal urine was $100 \pm 5\%$.

Experiments with rats fed with ^{35}S -labelled yeast

Samples of normal urine from rats fed with ^{35}S -labelled yeast and of urine from the same rats after dosing were acidified to pH 2 and extracted with ether. Samples of the two extracts, of the two urine residues and of the reference compounds were applied to sheets of paper. The spots were placed 2 in. apart. The developed chromatograms were dried and cut into 2 in. strips so that the spots were in the centres of the strips. The strips containing radioactive material were taped end to end and passed through a Packard chromatogram scanner with helium + butane (98.7:1.3) as the detector gas. The peaks obtained on the chart were compared with the position of the reference compounds (revealed by either $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 or the chloroplatinate reagent). The strips were then separated and photographed by exposure to X-ray photographic paper for 2 weeks. Finally these strips were sprayed with $\text{K}_2\text{Cr}_2\text{O}_7$ - AgNO_3 . The ethereal extracts of acidified urine from the undosed rats contained traces of radioactive material, but these were readily distinguished from metabolites present in the urines of dosed animals. No radioactive spots were obtained from either of the urine residues.

Experiments with $[1\text{-}^{14}\text{C}]$ styrene oxide

The urines of rats dosed with $[1\text{-}^{14}\text{C}]$ styrene oxide were examined as described above.

Hydroxylation in vitro

By tissue slices. The preparation of the digests of tissue slices was carried out as described by Bray *et al.* (1959). For each experiment three digests containing phenethylmercapturic acid (2 mg.), phenethylcysteine (2 mg.) and no substrate respectively were prepared. After the incubation the pH of the digest was adjusted to 5.0 with acetic acid and the mixture was homogenized in a Potter-Elvehjem-type homogenizer. The homogenate was heated to 70–75° to precipitate the protein, which was then removed by filtration and washed with water. The filtrate and washings were adjusted to pH 2 and continuously extracted with ether. The ethereal extract was examined for sulphur-containing compounds and a sample of the aqueous residue was concentrated and examined for phenethylcysteine.

Chemical. The ascorbic acid system of Udenfriend, Clark, Axelrod & Brodie (1954) was used. *N*-Acetyl-*S*-phenethyl-L-cysteine (20 mg.), ascorbic acid (150 mg.), EDTA (180 mg.) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (24 mg.) were incubated at 37° for 6 hr. with shaking. Samples of the solutions were examined by thin-layer and paper chromatography, and the remaining reaction mixtures were acidified to pH 2 and continuously extracted with ether. The extract was esterified with diazomethane and examined by gas-liquid chromatography.

RESULTS

Detection of sulphur-containing metabolites. The results of the examination of the ethereal extracts of acidified urines of dosed animals are given in Table 2. The results obtained by radioactive labelling are shown in Table 3. Radioautographs of the chromatograms of the ether extracts of the urine of rats that had been kept on a diet containing ^{35}S -labelled yeast and then dosed with phenethyl bromide showed a radioactive compound that did not react with the platinum reagent and gave with dichromate-silver nitrate a reddish colour barely distinguishable from the background. This compound is probably an ethereal sulphate of a metabolite of phenethyl bromide, since Bray *et al.* (1958) found that in the rabbit 13% of the dose of phenethyl bromide was excreted conjugated with sulphate. The compound ran with the β -hydroxyphenethylmercapturic acid in solvents A and D, giving an intense composite spot on the radioautographs; the corresponding spots revealed by the platinum and dichromate-silver nitrate reagents were much weaker and smaller. In solvent C β -hydroxyphenethylmercapturic acid was clearly separated from this compound, which had a rate of movement relative to phenethylmercapturic acid of 0.33. The amount of β -hydroxyphenethylmercapturic acid separated by solvent C was relatively small as judged by the size of the spot on the radioautograph and corresponded to that indicated by the detecting reagents in all three solvents and

Table 2. *Sulphur-containing metabolites in urine of dosed rabbits and rats*

The methods used for identification of the metabolites are indicated by the letters of the chromatography solvents (A, B, C and D for paper, and E and F for thin-layer) and by G for gas-liquid chromatography. —, Not detected.

Compound administered	Metabolites detected		
	Phenethylmercapturic acid	β -Hydroxyphenethylmercapturic acid	Unidentified metabolite
Phenethyl bromide	A, B, C, D, E, G	A, B, C, D, E, G	A, C, D
Styrene	Trace by E, G; not by A, C, D	A, B, C, D, E, G	A
Styrene oxide	—	A, B, C, D, E, G	A, C, D
Phenethylcysteine*	A, C, D, G	A, C, D, G	—
Phenethylmercapturic acid	A, C, D, G	A, C, D, G	—

* Phenethylcysteine also detected in urine by A and F.

Table 3. *Radioactive metabolites excreted in urine by rats*

Rats excreting metabolites labelled with ^{35}S had been given ^{35}S -labelled yeast in the diet and dosed with unlabelled compounds; those excreting metabolites labelled with ^{14}C had received normal diet and been dosed with $[1-^{14}\text{C}]$ styrene oxide. Unless stated otherwise compounds were detected on paper chromatograms developed in solvents A, B, C and D. No ^{35}S -labelled metabolites were detected in urine of rats given phenyl glycol. The number of + signs indicates approximate relative amounts of metabolites excreted after administration of a compound. —, Not detected. The same results were obtained in two experiments with each compound and three with phenethyl bromide.

Compound administered	Label	Metabolites detected		
		Phenethylmercapturic acid	Hydroxyphenethylmercapturic acid	Unidentified metabolite
Phenethyl bromide*	^{35}S	+++	++	+
Styrene	^{35}S	Trace†	++	+
Styrene oxide	^{35}S	Trace†	++	Trace
Styrene oxide‡	^{14}C	—	+§	—

* Solvents A, C and D only.

† By solvent A; not detected with B, C or D.

‡ $[^{14}\text{C}]$ Mandelic acid (equivalent to ++++) also detected. No radioactive spot reacted with naphtharesorcinol.

§ Solvents B, C and D only.

Table 4. *Amounts of metabolites excreted in urine by rabbits and rats*

Results are means expressed as percentages of dose, with ranges in parentheses; the numbers of determinations are indicated by superior figures. N.D., Not determined.

Compound administered	...	Phenethyl bromide*	Styrene	Styrene oxide†	Phenyl glycol	Phenethylcysteine‡	Phenethylmercapturic acid
Rabbit							
Dose (m-moles/kg.)	...	1.4	1.4	1.7	1.4	0.9	0.8
Glucosiduronic acid	...	20§ (15-33) ⁶	6 (4, 7) ²	20¶ (12-32) ⁷	24 (20-29) ³	N.D.	N.D.
Ethereal sulphate	...	13§ (9-15) ³	03	04	03	N.D.	N.D.
Hippuric acid	...	08	40 (37-44) ³	23 (15-32) ⁹	7 (4-13) ³	N.D.	N.D.
Mandelic acid	...	< 17	32 (27-36) ³	30 (25-35) ⁵	24 (17-29) ³	N.D.	N.D.
Apparent mercapturic acid	...	31§ (24-39) ⁵	5 (2, 7) ²	9 (8-10) ⁶	N.D.	N.D.	N.D.
Phenethylmercapturic acid	...	12.8 (11.3-15.0) ⁴	04	05	04	49.6 (43.0-55.5) ⁴	50 (42.0-55.5) ⁴
Hydroxyphenethylmercapturic acid	...	6.1 (3.0-8.5) ⁴	4.9 (3.0-6.5) ⁴	1.2 (0.7-1.6) ⁸	04	8.9 (6.7-12.3) ⁴	7 (4.5-12.5) ⁴
Rat							
Dose (m-moles/kg.)	...	1.0	2.0	2.1	1.3	0.8	0.7
Phenethylmercapturic acid	...	14.2 (12.9-16.3) ³	03	< 0.4 ³	03	50.0 (43.0-54.0) ³	76.3 (72.5-81.0) ³
Hydroxyphenethylmercapturic acid	...	1.9 (1.7-2.2) ³	8.5 (7.9-8.8) ³	5.9 (4.5-8.5) ³	03	< 1 ³	1.6 (1.0-2.0) ³

* Ether-soluble acid 79 (45-99)⁷ for rabbit (Bray *et al.* 1958).

† Ether-soluble acid 60 (50-75)³ for rabbit.

‡ Unchanged compound excreted: rabbit, 4.2 (3.0-5.5)⁴; rat, < 1³.

§ From Bray *et al.* (1958).

|| From El Masri *et al.* (1958) (mercapturic acid determined by iodine uptake).

¶ No increase in reducing value of urine was observed (three experiments).

to the quantitative results given in Table 4. The amount of the supposed ethereal sulphate was slightly greater than that of phenethylmercapturic acid. With all three solvents there was an un-

identified metabolite containing bivalent sulphur, present in smaller amount than the hydroxymercapturic acid.

Detection of phenols. No phenols other than those

present in normal urine were detected in the ether extract of acidified urine or of hydrolysed urine of rabbits dosed with styrene oxide.

Quantitative results. The amounts of metabolites excreted in urine by rabbits and rats are given in Table 4. Some results for rabbits already published have been included.

Experiments in vitro. With slices of rabbit and rat liver incubated with phenethylcysteine the formation of phenethylmercapturic acid and of hydroxyphenethylmercapturic acid was observed. When phenethylmercapturic acid was used as substrate phenethylcysteine and hydroxyphenethylmercapturic acid were formed. With slices of rat kidney the results were the same except that the hydroxymercapturic acid was not detected. The conversion was more rapid with rabbit than with rat tissue.

Chemical hydroxylation. The system of Udenfriend *et al.* (1954) brought about some conversion of phenethylmercapturic acid into the same hydroxyphenethylmercapturic acid as was detected in the experiments *in vivo* and *in vitro*.

DISCUSSION

The results show that in the rabbit phenethyl bromide is converted into phenethylmercapturic acid, as reported by Bray *et al.* (1958), and also into a metabolite chromatographically identical with synthetic *N*-acetyl-*S*-(β -hydroxyphenethyl)-L-cysteine. This hydroxymercapturic acid is also formed when styrene or styrene oxide is administered to the rabbit, and its excretion would account for the increased iodine uptake of alkali-hydrolysed urine of rabbits dosed with styrene reported by El Masri *et al.* (1958). No sulphur-containing metabolite was detected in the urine of rabbits dosed with phenyl glycol. Phenethylmercapturic acid was not detected in the urine of rabbits dosed with styrene oxide, but a trace was detected in the ethereal extracts of the acidified urines of rabbits dosed with styrene. When phenethylcysteine was administered to the rabbit, phenethylmercapturic acid and its β -hydroxy derivative were excreted with some unchanged material. Phenethylmercapturic acid administered to the rabbit was excreted largely unchanged, but some β -hydroxyphenethylmercapturic acid was formed.

Rats dosed with phenethyl bromide, styrene, styrene oxide, phenyl glycol, phenethylcysteine or phenethylmercapturic acid excreted qualitatively the same sulphur-containing metabolites as similarly dosed rabbits except that with styrene oxide a trace of phenethylmercapturic acid was detected in experiments with rats fed on ^{35}S -labelled yeast before dosing. In urines of both animals a trace of

an unidentified sulphur-containing metabolite was detected after dosing with 2-phenethyl bromide, styrene or styrene oxide. No evidence for a sulphoxide was obtained.

The quantitative results are not identical for rabbit and rat. The rabbit converts a greater proportion of the dose of phenethyl bromide, phenethylcysteine and phenethylmercapturic acid into the β -hydroxymercapturic acid than does the rat, but the proportion of the dose excreted as β -hydroxyphenethylmercapturic acid by rats dosed with styrene or styrene oxide is greater than that excreted by similarly dosed rabbits.

The specific method for the determination of mandelic acid shows that considerably more of this compound is excreted by rabbits dosed with styrene, styrene oxide or phenyl glycol than was found by El Masri *et al.* (1958), as would be expected since their results were based on the amount of mandelic acid isolated. Half the total ether-soluble acid excreted by rabbits dosed with styrene oxide consists of mandelic acid, but only a trace of this acid was detected in the urine of rabbits dosed with phenethyl bromide. The percentage of the dose of styrene oxide converted into hippuric acid was higher than that (9.5% of dose) reported by El Masri *et al.* (1958). No increased excretion of hippuric acid in rabbits dosed with phenethyl bromide was detected; this is in agreement with earlier results. The glucosiduronic acid excretion was raised in the urine of rabbits dosed with styrene oxide, but the nature of the glucosiduronic acid formed is not known. The increased excretion of glucosiduronic acid was not accompanied by the excretion of reducing material; no phenolic metabolite was detected in the acid-hydrolysed urine. This suggests the possibility of the formation of a glucosiduronic acid of an alcohol formed from styrene oxide, e.g. phenyl glycol, but there is no definite evidence to support this view. Against it is the failure to detect a radioactive glucosiduronic acid (by naphtharesorcinol) in the urines of rabbits dosed with [^{14}C]styrene.

Smith *et al.* (1954) reported that 15% of the dose of styrene oxide was conjugated with glucuronic acid. El Masri *et al.* (1958) failed to isolate and identify the conjugate and were unable to detect the glucosiduronic acid of phenyl glycol in the urine. In agreement with Smith *et al.* (1954) we detected no excretion of ethereal sulphate in rabbits dosed with styrene oxide.

The main features of the metabolic conversion of phenethyl bromide, styrene and styrene oxide into compounds containing bivalent sulphur are summarized in Scheme 2.

It is suggested that in both rabbit and rat phenethyl bromide (VII) reacts with GSH, the reactions being catalysed by glutathione *S*-alkyltransferase

Scheme 2. Metabolites of phenethyl bromide, styrene and styrene oxide. Reactions indicated by broken arrows were detected in traces only.

(Johnson, 1966), and that the conjugate so formed is broken down to give phenethylcysteine (VIII); this is acetylated to phenethylmercapturic acid (IX), which may then be converted into the β -hydroxy derivative (X). The conversion of phenethylcysteine (VIII) into phenethylmercapturic acid (IX) and its β -hydroxy derivative (X) and of phenethylmercapturic acid (IX) into its β -hydroxy derivative (X) has been shown to occur *in vivo* in both rabbit and rat. The quantitative results suggest that the conversion of phenethylmercapturic acid (IX) into its β -hydroxy derivative (X) occurs more readily in the rabbit than in the rat. It has been shown that liver slices of rabbit and rat convert phenethylcysteine (VIII) and phenethylmercapturic acid (IX) into the β -hydroxymercapturic acid (X) and that the hydroxylation occurred more readily *in vitro* with rabbit than with rat liver.

The main sulphur-containing metabolite of styrene oxide (XII) is the hydroxymercapturic acid (X), and the initial reaction in its formation might be catalysed by glutathione *S*-epoxidotransferase. Boyland & Williams (1965) demonstrated that styrene oxide is a substrate for this enzyme.

Styrene (XI) also gives rise to the hydroxymercapturic acid (X), and it is probable that styrene oxide (XII) is an intermediate. Traces of phenethylmercapturic acid (IX) are formed from styrene (XI) and styrene oxide (XII) in the rat, so that the hydroxymercapturic acid (X) might be formed from styrene (XI) and styrene oxide (XII) either by hydroxylation of phenethylmercapturic acid (IX)

or directly from styrene oxide (XII). The enzyme described by Boyland & Chasseaud (1966) might catalyse the initial reaction between styrene (XI) and GSH. It is unlikely that the formation of the hydroxymercapturic acid (X) from phenethyl bromide (VII) involves the intermediate formation of styrene oxide (XII) to any significant extent, since styrene oxide (XII) gives rise to relatively large amounts of hippuric acid (XIV) and mandelic acid (XIII), which are not appreciable metabolites of phenethyl bromide (VII). The possibility that the hydroxymercapturic acid (X) arises by hydroxylation of phenethylmercapturic acid (IX) has been demonstrated *in vivo* and with liver slices, and it has been shown that this conversion may be effected by a chemical hydroxylating system.

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